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METABOLIC FACTORS IN CARDIAC CONTRACTILITY

Consulting Editor

MENARD M. GERTLER

CONTENTS

This series of papers is the result of a conference on *Metabolic Factors in Cardiac Contractility* held by The New York Academy of Sciences on March 18 and 19, 1958.

INTRODUCTORY REMARKS

Menard M Gertler
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In recent years attention has been focused on the etiological factors contributing to chronic heart disease, that is, the hypertensive, atherosclerotic rheumatic, and congenital factors The terminal long-term problem (in the absence of sudden death) is often congestive heart failure For various reasons, it is difficult to know at the present time the exact incidence of congestive heart failure A hospital ward survey and a survey of 1000 consecutive private patients disclosed (1) 31 cases of grades 3 to 4 congestive failure in 180 ward beds, and (2) 93 private cases with grades 1 to 2 congestive failure (no attempt was made to classify the etiological type of heart disease) This terminal aspect of heart disease creates a high degree of morbidity in our population.

It is difficult to determine at present whether pathological structural changes in the myocardium precede biochemical changes, that is, physicochemical alterations that may result in ionic imbalance, microstructural aberrations, or protein loss that then produces a pattern of sequential events terminating in cardiac failure.

It is assumed, as a working hypothesis, that one of the principal and final causes of congestive heart failure is in essence a failure of the available chemical energy to be transformed and utilized as mechanical energy by the heart

The purpose of this monograph is to generate and promote new concepts concerning congestive heart failure that may be amenable to examination by techniques hitherto unused in this parameter In addition, it is thought that if the problem of congestive heart failure were presented by those familiar with the problem to individuals of other disciplines who were unacquainted with the problem, new approaches for study might become apparent Accordingly the conference on which this monograph is based was designed to bring together clinicians, physiologists, biochemists, biophysicists, and physicists in order that they might discuss the problem of congestive heart failure from their diverse viewpoints. The cross fertilization of such an approach has proved to be of inordinate value in other studies, and we hope that it will prove to be valuable in this field as well

IONS AND THEIR RELATIONSHIP TO HEART MUSCLE
A GENERAL SURVEY OF ION CONCENTRATION

George C. Griffith

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In 1954 I joined Butt and Walker of the University of Southern California School of Medicine in the publication of data on the concentrations of copper, iron, lead, mercury, manganese and zinc in various organs of the body. Our data had been obtained through spectrographic and chemical analysis of tissues taken at autopsy from 910 patients studied at the Los Angeles County Hospital. Investigation into the physiological and pathological significance of trace metals in man was getting under way at that time, and there was need for information on the normal levels of each metal in various body tissues, in order that we might go on to identify and investigate the deviant patterns occurring in diseased states.

The average amounts of copper, lead, manganese, and zinc found in hearts of patients in the age range of the fourth through the ninth decades are given in TABLE 1. Amounts are expressed in mg./100 gm. of dried tissue. Our current study does not embrace a sufficient number of hearts to establish norms for the other metals. Amounts of iron in these patients are much higher than the amounts reported in 1954, and we are forced to conclude that the earlier report was in error.

In a cooperative study now in progress in the pathology department and the cardiology section of the Department of Medicine, University of Southern California School of Medicine, we are seeking to determine whether conditions such as myocardial infarction or atherosclerotic heart disease are mirrored in changes in concentration of individual trace metals or in altered trace-metal patterns. Hegde, a young physician from India working in our section, has studied fifty hearts in the past year. In each instance he has obtained specimens from the right ventricle, the left ventricle, and the interventricular septum. Where coronary vessels have been involved, he has also obtained sections of myocardium including the involved arteries, and in special instances he has taken sections from the atria, the aorta, the endocardium, or the papillary muscles. A problem confronted us in determining which areas to sample. In the study of heart tissue from a patient with infarction of an area of the left ventricle, for example, was it necessary to sample both the infarcted and the noninfarcted area, or would the metal patterns of both areas be sufficiently similar that only one sample would be representative? We found that only the one sample was necessary unless both a new and an old infarct were present.

We used especial care in establishing our diagnoses. When the hearts were given to us by the autopsy surgeon, they bore only an autopsy number; we had no clue as to the diagnosis. Hegde removed the sections in which we

The work reported in this paper was supported in part by grant from Lakeside Laboratories, Chicago, Ill., the Orange County Heart Association, Santa Ana, Calif., and the San Bernardino Heart Association, San Bernardino, Calif.

TABLE 1

MILLIGRAMS PER 100 GRAMS OF DRIED TISSUE*

	Ages					
	31–40	41–50	51–60	61–70	71–80	81–
Copper	1 42	1 84	1 54	1 93	1 93	2 22
S E.	0 33	0 24	0 14	0 18	0 23	0 36
N	4	10	23	37	24	18
Lead	0 256	0 212	0 212	0 216	0 147	0 191
S E	0 068	0 036	0 038	0 040	0 022	0 034
N	7	11	31	43	31	20
Manganese	0 118	0 126	0 148	0 172	0 130	0 153
S E	0 017	0 012	0 028	0 024	0 008	0 022
N	7	11	30	43	31	20
Zinc	12 08	11 40	11 42	12 17	12 08	12 80
S E	1 05	0 97	0 39	0 78	1 12	1 70
N	7	11	30	44	31	20

* Average values
S E = standard error
N = number of cases

were interested, labeled them, and established the diagnosis histologically He then traced the patient s chart through the autopsy number and checked his findings against (1) the clinical impression of the patient s physician (2) entries on the chart itself and (3) the findings of the autopsy surgeon In most instances an electrocardiogram was available for confirmation of the diagnosis. In cases of suspected myocardial infarction, a serum transaminase study usually was available

Patients were grouped as follows (1) acut myocardial infarction (2) acut and chronic myocardial infarction (3) chronic myocardial infarction (4) atherosclerotic heart disease (5) idiopathic hypertrophy (6) endosclerosis and (7) amyloidosis.

We have just received spectrographic analyses of 13 metals in the first 32 f 50 hearts included in our study but we have not had sufficient time to make a thorough analysis of the findings and to check the clinical histories of patients whose metal patterns deviate from the normal However we have made a number of interesting and suggestive findings. Thirteen metals were studied in these hearts lead manganese, zinc, iron, copper molybdenum calcium aluminum cobalt silver nickel chromium, and cadmium

Lead content was extremely low in three patients, all f whom had greatly hypertrophied and flabby hearts with thickened left ventricles. A single patient with lead markedly elevated in his heart tissues proved to ha e a grossly enlarged right ventricular wall and huge l ver

A second trace metal studied, manganese proved most interesting When areas of recent infarction were analyzed the manganese concentration was found t be quite low but in areas contai ing older infarcts the concentrations of this metal were markedly elevated As an illustration in one heart with both an old and fresh infarct the manganese concentration at the site of t fresh infarction was only 0 03 mg /100 gm of dried tissue but in the

the old infarct the manganese value was as high as 0 29 mg (the average value for manganese in 143 persons of ages between 31 and 90 is 0 148 mg)

The concentrations of both zinc and aluminum were elevated in two patients with pulmonary edema plus intracranial hemorrhage and in a third patient with intracranial hemorrhage, but without any suggestion of pulmonary edema.

Chromium and nickel were lowered and silver was elevated sharply in a patient with epilepsy who had suffered a rupture of an aneurysm of the splenic artery and a lowered amount of cobalt was found in the heart of a patient with hemoptysis.

A 90-year-old woman with anomalous origin of the left circumflex artery markedly sclerosed coronary arteries, and heavy calcification of the mitral and tricuspid valves was found to have 506 mg calcium 100 gm of dried tissue in the tricuspid area and 388 mg calcium in the mitral valve area (the range of calcium in the hearts of our 32 patients in the current study is 5 8 to 160.30 mg the average value for the 3 measures)

For our initial studies, representative values were obtained by averaging the concentrations of the metal in the right ventricle the left ventricle and the IV septum As we go into more detail we plan to give attention to differences in metal concentrations and patterns in each area of the heart We are now trying to determine why in one patient with acute and chronic myocardial infarction, for example absence of detectable molybdenum is associated with normal levels of the other trace metals and in a second patient absence of detectable molybdenum is accompanied by absence of cobalt and chromium in the ventricles There are many such situations in the investigation, whatever metal we are studying

Interrelationship Between Metals Complex

Relationships of other trace metals to molybdenum serve to illustrate the interdigitation of the trace metals in the nutrition and metabolism of the body Most investigators have been aware for some time of the antagonism between copper and molybdenum especially in animal nutrition Copper deficiency in cattle may be due either to low copper concentration of pasture grasses or to high molybdenum content However a third factor enters into the situation, and perhaps a fourth The degree of molybdenum suppression of copper depends on the amount of inorganic sulfate in the diet and the combined inhibitory effect of the molybdenum and the sulfate on copper utilization seems to depend upon the amount of manganese available High manganese content will block the molybdenum-sulfate suppression of copper storage and utilization We must add further qualification If the diet is very high in protein, manganese may augment rather than block the effects of molybdenum and sulfur on copper utilization

Another trace metal that apparently interacts with molybdenum is tungsten, which is known to inhibit the action of intestinal xanthine oxidase.

Metals and Arterial Hypertension

Perry and Schroeder[7] have pioneered in elucidating the roles of the trace metals in arterial hypertension After the discovery that hydralazine an

antihypertensive agent selectively binds manganese ferrous iron, copper silver tin and mercury and that hydralazine is oxidized with the liberation of nitrogen by vanadium and ferric iron, these investigators tested a number of chelating and metal-binding agents and found that several would lower arterial hypertension. They then analyzed the chemical structures of ant hypertensive substances now in use or undergoing clinical trial, and found that many apparently dissimilar agents, none of which act on the autonomic nerves, had the common property of binding trace metals

Occasionally persons treated with dimercaptopropanol (BAL) or ethylene diaminetetraacetate (EDTA) two chelating agents that have been used to lower hypertension, respond paradoxically with a further elevation in blood pressure [18] This suggests that mobilization of metals can affect vascular homeostasis in either direction

Schroeder *et al* [17, 18] have isolated a peptide, pherentasin, from the arterial blood of hypertensive patients Pherentasin is inactivated by each of six metal-binding or chelating agents, providing evidence that the constriction of vascular smooth muscle may be influenced by a metallopeptide in the blood

Cadmium, lead, and manganese are found in increased amounts in the urine of persons with malignant hypertension [14] This suggests that these metals may be present in hypertensive patients in abnormally large amounts As might have been predicted when hypertensive patients are treated with agents such as hydralazine, high urinary levels of these metals drop toward normal Excretion of one metal, vanadium, is considerably less in hypertensive patients than in normal persons Only after a large series of cases has been thoroughly studied will it be possible to determine the role of metalloenzyme imbalance in arterial hypertension and to apportion with certainty the contribution of each trace metal to that imbalance. In our laboratory we shall be interested in metal assays of tissue samples from the hearts of hypertensive patients in our series

Metals in Atherosclerosis

Metals also may play a part in atherosclerosis. We have evidence that metals are intimately involved in fatty acid metabolism For example, Curran has shown that in rats chromium increases by 150 per cent the incorporation of C^{14}-labeled acetate into cholesterol and fatty acids. Manganese long recognized as a lipotrophic agent increases synthesis by 125 per cent vanadium decreases synthesis by 90 per cent The introduction of the chelator 8-hydroxyquinoline decreases cholesterol and fatty acid synthesis, suggesting that different metals are mobilized or removed by the metal-binding agent

Metals in Rheumatic Fever

The detection of zinc coproporphyrin in the urine in acute rheumatic fever suggests that zinc may be involved in this heart-crippling disease [17] Often the auricular appendages removed routinely during mitral commissurotomy show signs of active rheumatic disease We have been saving these tissues and plan to assay their trace metal content by means of the emission spectrograph

Metals in Congestive Failure

We are learning more and more about the roles of ions other than sodium in the pathogenesis and control of congestive failure. In the clinic conducted at the Los Angeles County Hospital we have maintained patients free from significant renal disease for months or years on moderate or massive doses of organomercurials without injury to kidney function, provided neither oliguria nor hyponatremia is present. Analysis of the kidney tissue from 45 patients with congestive failure showed an increase of mercury deposit in the kidney with continued administration, but gave no evidence of interference with kidney action. Nonprotein nitrogen values for these patients could not be interpreted as indicating that the larger amounts of mercury in the tissues were harmful.

Less well known is the method of action of carbonic anhydrase inhibitors. Here, too, a metal is of primary importance, for carbonic anhydrase inhibitors work by blocking the action of zinc, the metal that is an indispensable part of the carbonic anhydrase molecule. Removal of the zinc results in irreversible inactivation of the metalloenzyme; reversible inactivation is effected by enzyme inhibitors that combine with zinc, such as dimercaprol, cyanide, sulfide, azide, and the sulfonamides.

I shall not discuss other diseases affecting the cardiovascular system in which metals may play important roles, but I shall outline the paths our own investigations will follow.

(1) We shall make a thorough study of the metal concentrations in the hearts now under study and shall try to find an explanation for abnormal metal concentrations or unusual trace-metal patterns in the patients' clinical histories.

(2) Hegde also has preserved additional tissues from the various chambers of the fifty hearts under study. These tissues will be studied for enzyme patterns, which will be compared with the tissue metal profiles.

(3) In most instances the patients in our series with acute or chronic myocardial infarction have had serum transaminase studies in the period before death. Comparisons will be made between the serum transaminase studies and tissue enzyme patterns.

(4) We have made arrangements to inject radioactive-tagged manganese into patients with myocardial infarction and thus to follow the incorporation of the tagged manganese into serum cholesterol.

(5) As I mentioned previously, by means of emission spectroscopy we plan to study trace metals in the auricular appendages of persons undergoing surgery for mitral commissurotomy.

When we first undertook the study of metal concentrations in cardiovascular and related tissues in the early 1950s, I was convinced that we had in the metals a tool that would permit us to diagnose and treat pathological conditions long before these conditions had advanced to the point where they could be detected by means of the slide and the microscope. Developments have been even more rapid than I envisioned, and evidence is mounting that metals hold the key to an entirely new dimension of medical investigation that will one day enable the clinician to treat disease with the accuracy expected of the chemist or the physicist.

Acknowledgment

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MAINTENANCE OF CONCENTRATION GRADIENTS AND REGULATION OF CELL VOLUME

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It has long been recognized that a high surface-to-volume ratio is important to rapidly metabolizing cells that are dependent on diffusion for supplies of oxygen and nutrients from the surrounding medium. It is also well known that the cell has a high protein content, a significant proportion of which exists in a soluble state in which it may be expected to exert an oncotic pressure. The interstitial fluid bathing the cells, however, is relatively low in its content of colloids. From studies with isotopically labeled tracers over the past twenty years it is now well established that the cell membrane is permeable to water and to all the solutes that contribute significantly to the osmolality of the extracellular fluids. These solutes are largely the salts of sodium.

With these facts in mind one might expect that the oncotic pressure exerted by the intracellular proteins would cause disastrous swelling by drawing fluid from the extracellular compartment into the cells. Such swelling of cells obviously does not occur in healthy tissues *in vivo*. One is therefore justified in inquiring into the nature of the forces that normally counteract this tendency toward swelling of body cells and thereby preserve the normal intracellular fluid volume.

The maintenance of normal intracellular volume has been shown to be a process requiring energy. In 1949 Stern *et al.* observed that the swelling of various tissues *in vitro* was dependent upon tissue respiration. They tested the effect of aerobic versus anaerobic incubation in an isotonic saline medium on the fluid content of various tissues of the guinea pig. Whereas only small changes in weight occurred during aerobic incubation, anaerobic conditions resulted in a consistent large weight gain attributable to an increased water content of the tissue. This type of observation has been repeated by a number of other workers using various tissues and various means of inhibiting metabolism. Mudge, Robinson, Deyrup, and Whittam and Davies[1] have all repeatedly confirmed this finding using anaerobiosis, chilling, dinitrophenol, azide, and other methods of interfering with tissue metabolism. In all instances inhibition of metabolism has been found to be associated with increased water content of the tissue studied.

A much older observation that goes back at least to Sabbatani's work at the start of this century is that swelling of tissues *in vitro* can be prevented by substituting hypertonic solutions for the usual isotonic saline media. Gomori and Molnar, Opie, Robinson, Aebi, and others have confirmed this finding.

The observations that tissue swelling is dependent upon tissue metabolism

The work described in this paper was supported in part by funds from The John A. Hartford Foundation, Inc., New York, N. Y., from the National Heart Institute (Grant H 2822), Public Health Service, Bethesda, Md., and from the American Heart Association, New York, N. Y.

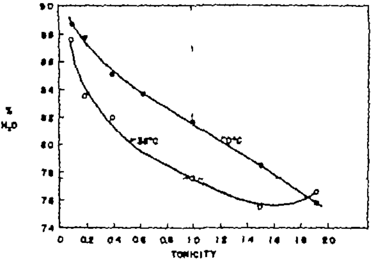


FIGURE 1 The relationship between water content of tissue slices and concentration of the incubating medium This figure is a replot of data of Robinson On the abscissa is shown concentration of the medium expressed as factors of 0 15 M saline and on the ordinate is the water content of the tissue (rat kidney cortex slices) expressed as per cent of wet tissue weight The intersection of the two perpendicular broken lines indicates the normal water content of metabolizing tissue in an isotonic medium The lower curve shows the changes in concentration of the medium for metabolizing tissue incubated at 38° C, whereas the upper curve represents the water content of tissue whose metabolism was inhibited by incubation at 0° C

and that it can be prevented in hypertonic media have given rise to the concept that the intracellular fluids have a higher osmotic pressure than that of the extracellular fluid and that this osmotic gradient is preserved *in vivo* and in metabolizing tissue slices *in vitro* by an active extrusion of water from the cell Such an active extrusion of water to maintain cell volume seemed to be a necessary requirement as studies with isotopically labeled water have established the permeability of cell membranes to water

The experimental facts upon which this concept has been established are summarized in FIGURE 1 which was constructed from data of Robinson On the abscissa is plotted concentration of the medium expressed as factors of 0 15 M saline and on the ordinate is shown the water content of the tissue (rat kidney cortex slices) expressed as percentage of wet tissue weight The point of intersection of the 2 perpendicular broken lines indicates the normal water content of metabolizing tissue in a medium isotonic to extracellular fluid The lower curve shows the changes in water content resulting from changes in concentration of the medium for metabolizing tissue incubated at 38° C whereas the upper curve represents the water content of tissue whose metabolism was inhibited by incubation at 0° C

Inhibition of metabolism without change in tonicity of the medium is seen to produce a large increase in tissue water content Over a considerable range of concentration, the water content was found to be higher when tissue metab

TABLE 1

CHANGES IN THE SODIUM, POTASSIUM, CHLORIDE, AND WATER CONTENTS OF GUINEA PIG KIDNEY CORTEX SLICES ON INCUBATION AT 0° C

Slices of Tissues (0.1 to 0.2 gm.) Incubated 30 to 50 min. in 2.0 ml. of Bicarbonate Saline, pH 7.4, Gas Phase $O_2 + CO_2$ (95:5)†

	No. of samples	Dry wt. per cent	Water per kg. dry solids (kg.)	mEq./kg. dry solids		
				Na	Cl	K
(a) Before incubation	14	23.7 ± 0.35	3.22 ± 0.04	350 ± 16	272 ± 7	365 ± 8
(b) After incubation	14	19.4 ± 0.29	4.16 ± 0.05	579 ± 18	417 ± 17	240 ± 7
b − a	—	—	+0.94 ± 0.06	+229 ± 24	+145 ± 18	−125 ± 11

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† Mean values ± S.E.

olism was inhibited than when the tissue was metabolizing. Furthermore, an increase in concentration of the medium, in this case to 1.65 times isotonic, was effective in preventing tissue swelling even though metabolism was inhibited. As stated, these findings have been interpreted as evidence for a higher intracellular than extracellular osmotic pressure that is maintained in metabolizing tissue by the active extrusion of water as rapidly as it enters the cells.

This argument is supported by the evidence thus far presented only if the tissue swelling is due to net shifts of water *alone* into the tissues. If net movements of osmotically active solute also occur across cell membranes during such *in vitro* studies, such an interpretation is not justified.

TABLE 1 shows the effects on the water, sodium, potassium, and chloride content of 0.1 to 0.2 gm. slices of guinea pig kidney cortex when incubated 30 to 50 min. at 0° C. in a saline medium similar to medium 3 of Krebs[1] without addition of the organic substrates. Control slices were unincubated, fresh slices of tissue. Results are expressed per kilogram of tissue solids. The tissue swelling is indicated by the increase in tissue water of 0.94 liters. The increase in tissue sodium and decrease in tissue potassium noted by others is seen. Of perhaps more importance to the present argument is the large increase in tissue chloride that occurred. One can calculate the concentration of chloride in the increment of water gained: 154 mEq./l. As the concentration of chloride in the incubating medium was 135 mEq./l., the data strongly suggest that the gain in tissue water content in these experiments represented essentially an isotonic entry of medium into the tissue. This finding invalidates the assumption that tissue swelling is due only to net movements of water between tissues and medium.

Similar results have been obtained when other mammalian tissues have been studied and other means of inhibiting tissue metabolism utilized.[11] These results confirm studies by Mudge, who had previously shown that the tissue swelling produced by a variety of inhibitors of metabolism was associated with an uptake of ions as well as of water by the swollen tissue. Our findings are

fully in accord with his conclusion that "depression of metabolic activity is, therefore, associated with an isosmotic increase in cellular hydration."

The gain in tissue water and solute in the present experiments is probably shared by both the intra- and extracellular phases of the tissue. Thus, Robinson[4] found by measurement of the inulin and sucrose spaces in slices of rat renal cortex that the "extracellular" water remains an approximately constant fraction, 26 per cent, of the weight of the slices whether tissue hydration is normal or increased. This finding has been confirmed with slices of guinea pig kidney cortex incubated aerobically at 0° C. by Whittam.[12]

From the above it is apparent that the tissue swelling that occurs when metabolism is inhibited results from a movement of both solute and water into the tissues. This invalidates the argument that it is due to water alone moving in response to an osmotic gradient. This type of evidence cannot, however, provide an answer to the fundamental question of whether a gradient does in fact exist between the water activity of the extracellular fluid and that of the fluid within the cells. To answer this question, we must turn to a more direct experimental approach.

In 1901 Sabbatani sought to determine the freezing point of various dog tissues and obtained values that averaged about 150 per cent greater than that for blood. Collip,[13] Leovey and Kerpel-Fronius,[14] Gomori and Molnar, and Pitchotka *et al.* all confirmed Sabbatani's findings, as did Opie[1] using melting point depression. These cryoscopic measurements have been criticized, however, because autolysis occurring during the interval between removal of the tissue from the animal and the end point of the measurement will yield spuriously high values for tissue tonicity. That a rapid increase in freezing point depression of tissues will occur even at 0° C. has been demonstrated by Conway and McCormack,[17] Conway *et al.*,[18] and by Brodsky *et al.* Conway and McCormack[17] claimed that the freezing point depression of tissue, corrected for the expected autolysis, was the same as for the serum. Brodsky *et al.* were unable to confirm these extrapolated values. However, by heating tissues in water at 98° C. for 30 min. immediately upon removal to reduce autolysis and then measuring the freezing point of the heated mixture, Appelboom[19] has recently reported isotonicity with extracellular fluids.

In our laboratory LeRoy H. Maffly recently has utilized a new technique for obtaining tissue-melting curves. Mammalian tissues were frozen by immersion in liquid nitrogen immediately upon removal from anesthetized animals. At the temperature of liquid nitrogen the tissue was pulverized and the finely ground powder suspended in an inert, chilled, liquid silicone. This yielded a liquid suspension of tissue that could be stirred vigorously and warmed gradually in the presence of a sensitive temperature measuring device. By this means, tissue autolysis was avoided, as were thermal gradients in the thawing tissue. Details of this study will soon be published elsewhere, but FIGURE 2 shows the results Maffly has obtained for cardiac muscle of the rat. Here are plotted for comparison the terminal portion of the melting curves of rat heart, normal rat serum having a concentration of 311 mOsm./l. (as measured by an independent precise freezing point method), serum concentrated by dehydration to 508 mOsm./l., and serum diluted to 100 mOsm./l. The ordinate

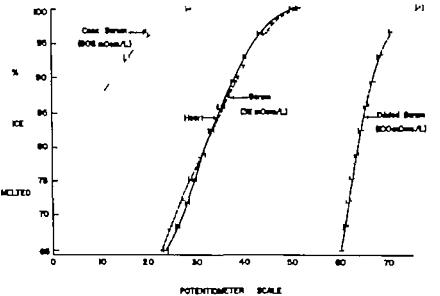


FIGURE 2 Melting curves for rat heart (ventricular muscle) compared with those of normal rat serum and concentrated and diluted rat serum Measurements are made on portions of tissue or serum containing equal total quantities of ter The ordinat is the percentage of this ter that has melted and the abscissa is the potentiometer reading hich is proportional t temperature or total solut concentration The horizontal distance between the curves is measure of the difference in solut concentration or of water activity between the respectiv curves Each curve represents the mean of 12 measurements and the small vertical bars or row of dots show the standard error of the mea for each point plotted It is evident that there is no difference between the measurements for heart muscle and for serum

the percentage of ice, in tissue or serum, that has melted and the abscissa is the potentiometer scale proportional to the temperature of the melting suspension or to its osmolality Each curve is the mean of 12 separate measurements. The standard error of the mean for each point is shown by the small vertical bars or rows of dots. It is evident that the values for heart muscle do not differ significantly from those of the serum Similar results ha e been obtained by Maffly for skeletal muscle, liver brain, and kidney cortex of the rat

These direct measurements prove conclusively that the water activity within the cells is the same as that of the extracellular fluids and that no osmotic gradient exists between the intracellular compartment and the interstitial fluid. What then, is the force that causes tissues to swell when their metabolism is inhibited and how is this swelling prevented *i vivo* and in metabolizing tissues *in vitro*? I propose one view that is consistent with current knowledge of ion concentrations in tissues

It has long been recognized that the fluid contained within cells is high in potassium and low in sodium and chloride concentrations while in the extra cellular fluids, this relationship is reversed that is, sodium and chloride ions are present as the major solutes while potassium exists at a low concen

tration Since studies with isotopes have demonstrated that the cell membranes are permeable to sodium, potassium, chloride, and water it is evident that maintenance of the ion gradients by tissues requires the expenditure of energy Specific active transport systems have been described for sodium,[illegible] potassium,[illegible] and chloride[illegible] in various cells and tissues. Whether these transport processes operate singly or together in all cells to extrude sodium and chloride and to accumulate potassium in order to maintain the characteristic ion gradients of each tissue is still undetermined The observed ion gradients in many tissues could simply result from an active outward extrusion of sodium alone from the cells, with other ions following passively.[illegible] Recent studies indicate that the actual situation is more complex than this[illegible] and suggest an active transport process for at least potassium as well as sodium.[illegible] In all instances, however cells utilize energy derived from metabolism to operate their ion pumps

The interior of most cells is electrically negative relative to the exterior of the cell The electrical polarity of the cell membrane is attributed to the content in the cell of fixed or nondiffusible anionic groups on intracellular proteins and other large molecules and to the outward extrusion of sodium One effect of this electric potential across cell walls is to contribute to the exclusion of chloride and other negatively charged small ions of the medium The intracellular negativity electrostatically opposes the like negative charge of chloride ions

When metabolism is inhibited, the energy required for the ion transport process is no longer available Sodium that diffuses down its concentration gradient into the cells can no longer be extruded potassium leaks out of the cells into the surrounding extracellular fluid As a result of the accumulation of sodium in the cell and loss of potassium, the membrane potential is reduced so that chloride as well as sodium enters the cell This results in a greater entry of sodium than loss of potassium by the tissue as shown in TABLE 1 The net gain of solute by the tissue is accompanied by an isotonic gain of water and the tissue becomes swollen † The process is reversible—for a time at least—so that resumption of metabolism causes the high fluid content of the swollen tissue to return toward normal [illegible]

Lillie demonstrated in 1907[illegible] that the osmotic pressure exerted by a given concentration of protein decreases with increasing concentration of neutral salts in the medium It is theoretically possible therefore that swelling of tissues may be prevented even when metabolism is inhibited by placing them in hypertonic salt solutions This as shown in FIGURE 1 is exactly what Opie Robinson, and Aebi have found experimentally It is clear from what has been stated above however that this finding does not justify the claim that

* For a more rigorous description of the obligatory entry of chloride with sodium into cells when metabolism is inhibited, see Leaf

† An increased permeability of the cell membrane to sodium may contribute to the intracellular accumulation of this ion when metabolism is inhibited An increased influx of sodium into the cell could cause swelling even when the outward extrusion of this ion was not completely inhibited As yet, not enough is known about the factors which control cell membrane permeability to permit speculation regarding this additional variable

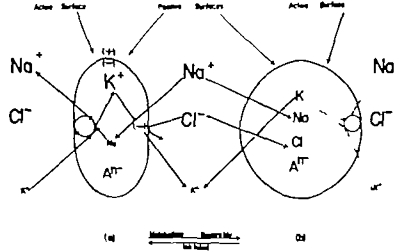


FIGURE 3. Schematic representation of normally metabolizing cell ( ) and cell whose metabolism has been reversibly inhibited (b) *to illustrate the important factors regulating* cell volume

(a) In the normally metabolizing cell Na diffuses into the cell and K out, as shown on its right hand side. However active ion transport indicated by the rotating wheel on the left margin of the cell, extrudes N and causes K uptake sufficient to maintain high intracellular K content and low intracellular Na content, respectively, in the steady state. The cell membrane potential is shown (+) outside and (−) inside. The membrane potential is depicted as preventing entry of chloride into the cell. A^{-} represents nondiffusible polyvalent intracellular anions (such as proteins and nucleic acids)

(b) The situation in swollen cell is depicted. Inhibition of metabolism slows or stops the ion pump so that the N entering the cell by diffusion cannot be extruded and the K lost from the cell cannot be replaced. The cell membrane potential diminishes and chloride now enters the cell. This requires further entry of N into the cell to preserve electric neutrality. The increased solute content of the cell results in secondary entry of water and the cell becomes swollen. Whether changes in cell membrane permeability to passive entry of ions also occur when metabolism is inhibited and contribute to the entry of solute into the cell is as yet unknown

the high concentration of such media represents the true total solute concentration within metabolizing cells

FIGURE 3 summarizes schematically the view presented here. FIGURE 3a shows a normally metabolizing cell of usual volume. The content of nondiffusible intracellular material, largely protein with net negative charge at physiological pH, is indicated by A^{n-}. An active transport mechanism is depicted as a wheel rotating in a clockwise direction to extrude sodium from the cell and to take up potassium from the medium at a rate sufficient to maintain steady-state concentrations of these small ions in spite of a slow passive diffusion of sodium into the cell and leakage of intracellular potassium to the medium. The relative concentrations of ions are roughly indicated by the size of the letters. The oncotic pressure within the cell is thus offset by the obligatory extracellular position of sodium, which is maintained at the expense of energy from tissue metabolism. A double Donnan system is set up, and swelling does not occur

FIGURE 3b shows the effect of inhibiting metabolism. The activity of the ion pump is reduced so that it can no longer extrude sodium or take up potassium as rapidly as sodium diffuses down its concentration gradient into the cell or as potassium is lost from the cell. The result is a net gain of osmotically active solute within the cell and the cell swells. A return of metabolism will result in sodium extrusion and potassium uptake with return to the unswollen normal state.

Finally I make one further speculation. What is the significance of this mechanism to the cell?

The alternative adjustment possible to the cell to prevent the swelling that would result from its content of intracellular colloid would be to surround itself with a rigid casing to withstand this force. However, a high membrane tension would be very disadvantageous to small cells, which are dependent for many vital processes on a high surface volume relationship. Furthermore, in those instances in which membrane tensions of animal cells have been measured, very low values have been obtained. E. N. Harvey[36] summarized this finding by stating "We are accustomed to thinking in terms of air-water surface tensions of 73 dynes/cm so that it is hard to realize that the tension of cells is at least 1000 times, perhaps 10,000 times less." These low membrane tensions are possible, I believe, because the animal cells have learned to avoid disastrous swelling by developing the ion pumping mechanism just discussed. Certain cells seem to have evolved secondary uses for the energy stored in the ion gradients or in the resulting cell membrane potentials. Nervous conductivity and muscular contractility, perhaps, represent examples of such secondary evolutionary adaptations. Thus we owe the contractility of the myocardium and, *in fact, our very mobility as compared with the sessile members of the vegetable kingdom*, to this method of preserving cell volume which avoids the cumbersome cellulose casing that the plant cells chose as their answer to the threat of cell swelling.

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It has been shown that rapid autolysis may occur in tissues upon removal from the body.[9] Such changes could result in increased intracellular osmotic pressure with resultant tissue swelling. The results in TABLE 1, however, indicate that sufficient extracellular solute entered the swollen tissue to account for the gain in tissue water without the need for any additional source of solute in the tissue. Furthermore, the process of swelling discussed here is reversible. For these reasons the possible minor contribution of autolysis to the problem of swelling is unmentioned in this presentation, but not denied.

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PHYSIOLOGICAL EFFECT OF IONS

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Introduction

At one stage in the development of our modern concepts of renal function a well-known physiologist said, "All we know *for sure* about the kidney is that it makes urine." Although the literature abounds with ingenious theory and clever painstaking and even thoughtful experimentation regarding the effects of ions on cardiac function, one is likely to conclude upon perusal of the material that "All we know *for sure* about the physiological effects of ions is that there are some." It should be added, in haste, that following the quoted utterance on kidney function much has been learned—enough, indeed, to bring on the equally famous remark that "... the kidney is the only *thinking* organ in the body." Although, as we shall see, our present position regarding ion effects is precarious, we thus have excellent reason to hope that similar progress will occur in relation to this problem.

Reviews on ionic disposition, ionic concentration (both actual and relative) and ionic movement, and theories of ionic effects in a wide variety of animal forms and tissues abound. With the combined advent of isotopic and flame photometric methods, the research reports in this field are rapidly increasing in both number and value. Thus, the task of reviewing even a limited portion of the field is monumental.

Cardiac Ion Disposition and Membrane Potentials

The intracellular phase of heart muscle, like most animal cells, has a higher K and a lower Na and Cl concentration than is present in the extracellular fluid, whereas Ca appears in approximately equivalent concentrations inside and outside the cell. The functional barrier that provides for these distributional inequalities is probably not the anatomical cell border but rather the subcuticular layer of the cell protoplasm, the properties of which appear to differ from those of the contiguous cell interior.

The importance of this limiting or partially limiting membrane in effecting ionic partition during both rest and activity makes a brief description of its probable structure germane. It is assumed from comparative X-ray diffraction studies, from water solubility measurements, and from surface tension considerations that a few layers of protein molecules probably overlie a thin lipoidal material—the combined system being of the order of 200 Å in thickness. Whatever the intimate structure, however, the resting potential is certainly related to the differences in ionic concentration on the two sides of the functional barrier. Proceeding from the potential difference across the resting cardiac fiber membrane (0 to 100 m) and from what we know or can surmise regarding the ionic concentrations of the intracellular phase, we can infer that the membrane is more permeable to ions than t anions. Similarly, it

can be seen that while K^+ and Cl^- move *with* the potential gradient (but in opposite directions) Na^+ leaving the fiber must be transported *against* both a concentration and an electric gradient

Most of the statements usually made regarding ion disposition and movement are only partially true and are based upon net results of movements in both directions, the dynamics of which are only slightly understood Nevertheless, it has been necessary to posit some theory as to the mechanism whereby an ion can be transported against an electrochemical gradient, and it is recognized that in the final analysis such a "pumping" mechanism must depend upon energy yielding metabolic events within the cell Such theories may be necessary to explain inflow of K ions as well as the ejection of Na ions, although no firm evidence for the former (as far as the heart is concerned) has yet been produced

Assuming the foregoing to be approximately true, it is clear that the imposition of external current flow (action current) or any effective reduction in the metabolic activities governing membrane permeability would result in lowering the resting potential to the point of allowing a sizable Na influx Whether a concomitant depression in Na expulsion occurs is not known but in any case, the extrusion mechanism for Na is temporarily overcome and the polarity is reversed Thus the rising phase of the cardiac action potential appears to be the result of the entrance of Na ions int the cell The falling phase, on the other hand, is apparently the result of (1) a recovery of effectiveness of the Na extrusion mechanism (or reduction in Na permeability) and (2) an increase in membrane permeability to K bringing about a net outflow of positive charge and tending to restore the resting potential However the phenomena associated with repolarization of cardiac membranes are less well established than those accounting for depolarization The fact that the resting potential is not re-established for about 200 to 300 msec indicates that in heart muscle either the outflow of K^+ is delayed or the alteration of the membrane toward again actively extruding Na^+ is slow relative to the conditions found in nerve It has been suggested that part of the recovery phase is due to metabolic action of the mitochondria,[4] the membrane properties of which seem to be such that Na can be held against a concentration gradient

Temperature Effects Electric Events and Ionic Disposition

Trautwein *et al* and, more recently Marshall and others have shown quite consistent changes in the membrane potentials of isolated atrial fibers subjected t cold In general, these changes consist of a reduction in the resting potential the most striking reduction appears at temperatures below 20° C., but definite downward trends are shown even at 25° C The results of cooling have been so consistent indeed that there has been considerable speculation that such potential redu tions are the result of cold depression of the metabolic phenomena responsible for either Na^+ outflow and/or K^+ inflow Conclusive evidence of these assumed ionic events f r the isolated fiber preparation is not available, however

Studies on the effects of cooling the whole animal on cardiac ion distribution have been made. [13] Some of these investigations utilize coronary arterio-

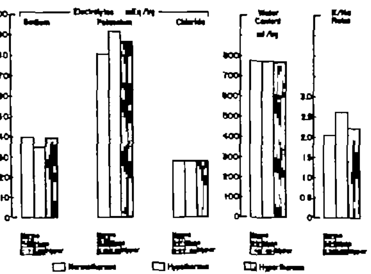


FIGURE 1. Average left ventricular Na, K, Cl, and H_2O per kg wet tissue and the K/N ratios for 30 normothermic dogs, ten animals cooled to 25° C for 2 hours, and 10 dogs heated t 41.5° C for 1 hour. The enclosed numbers beneath the bars are *t* values for the comparisons indicated at the top and side of the boxes. The boldface numbers designat statistically significant differences, $P \leq 0.05$.

venous differences (without knowing coronary flow) in order to arrive t possible or probable changes in heart ion disposition in hypothermia. Others have employed the "stabilized" tracer technique but have often neglected to measure total tissue ions. Few groups have maintained animals at hypothermic level for a period sufficiently long to have been studying a steady-state situation. Recently Spurr *et al.*[13] have maintained animals at 25° C for a 2-hour period prior to analysis of ventricular muscle for Na, K, Cl and water. At the time of taking cardiac tissue for analysis, these animals had revealed few electrocardiographic abnormalities other than a raised S-T segment. A portion of the results obtained is shown in FIGURE 1. It will be seen that, contrary to expectations raised by studies of electric responses of cooled single fibers and, in general, contrary to other studies of the whole animal in which animals were cooled to about 25° C and immediately sampled, the results of this study indicate a significant reduction in ventricular muscle Na and a significant rise in K. There are no changes noted in Cl concentration nor in water content of the muscle.

Whereas the single fiber studies are excellent presentations of electric events and membrane potentials without accompanying ionic data, the whole animal studies thus far—revealing as they are of chemical anatomy and even of ion dynamics—have failed t present simultaneous electric patterns either of static charges or activity potentials. Weidmann[14] has previously pointed out the desirability of obtaining data "that allow distinguishing between turnover in the resting heart and the increment or decrement due to activity." It probably equally important to make studies in the steady state to take

able account of pH alterations, to study multiple ion movements all in the same preparation, and to attempt to correlate such findings with electric events or at least, with potential differences.

The paucity of information regarding low temperature effects on ion distribution in the heart is exceeded only by that relating hyperthermia to ionic movement and electric activity. FIGURE 1 indicates that in the steady hyperthermic state (41.5 C for one hour) ventricular muscle gains no Na^+ but shows a K^+ rise that while less than that of the hypothermic heart is yet significantly different from control values. Again however intimate knowledge of simultaneous electric events (other than external EKG) is lacking.

Changes in Concentration of "Extracellular" Ions

It is generally conceded that increasing the extracellular K concentration produces a shortening and a reduction in magnitude of cardiac action potentials, as well as a decrease in the membrane potential. Perfusion with a K free solution on the other hand, increases the resting potential as well as the duration of the action potential. Weidmann[1] has shown that, in the isolated turtle ventricle, increasing the K concentration in the perfusate causes transient depolarization of the resting fiber whereas such an extracellular increment of K produced an earlier and incomplete repolarization of the same fibers when stimulated. The value of the resting potential then, is assumed to depend upon the ratio of intracellular K to extracellular K. However the repolarizing effect of K in the active preparation must be explained either on the basis that an increase in outward K current has occurred or that there has been a decrease in inward Na current, or that somehow the rise in extracellular K has stimulated the Na pump and thus increased Na extrusion.

Whereas the influence of K is limited primarily to the establishment or maintenance of a given membrane potential and to the repolarization process in an active fiber Na is principally concerned in the depolarization process. Thus, increasing extracellular Na concentration leads to a higher action potential and a greater overshoot "hyponatremia" brings about the reverse. Insofar as Li can replace Na (up to approximately one third of the external concentration) it acts as does Na. In higher concentrations the effects of Li are more like those of decreased extracellular Na concentration except that it shortens the duration of the action potential. The primary effects of Ca (which are understood) are those of altering the threshold and of antagonizing or reversing the changes brought about by K.

Effect of "Normal" Inotropic Agents on Cardiac Ion Partition

Recently Burn[1] reviewed the paradoxical action of acetylcholine on atrial tissue and related these phenomena to ionic distribution and to changes in membrane permeability to ions. The normal inhibitory action of acetylcholine and its effect on increasing the polarization of the atrial cell are well known. If atrial tissue is cooled to somewhat less than 20° C contractions cease and action potentials are no longer propagated in the peripheral atrial tissue. Under these conditions, however small, rhythmic, nonpropagated potentials

are still present in the pacemaker and, if acetylcholine is added (after a brief delay) regular normal impulses again appear in the peripheral atrial musculature, and contractions are resumed. If the pacemaker impulses also disappear acetylcholine is without effect and, therefore, it cannot be acting by depolarizing the membranes. These findings have been confirmed and extended by Marshall. Similarly if the atria are allowed to beat until K loss lowers the resting potential to the point where peripheral atrial action potential propagation is unsupported, the addition of acetylcholine restarts contraction. It can be shown also that vagal stimulation acts in exactly the same way and, further that the excitatory action of the vagus under these conditions can be blocked by atropine.

As previously stated, the action of acetylcholine is not one of fiber depolarization, but rather of alteration of atrial cell permeability to K ions, thus raising the resting potential Hoffman and Suckling[u] ha e shown that acetylcholine accelerates repolarization in atrial tissue and, as K is largely associated with the repolarization process, this would seem t be additional evidence of the fact that acetylcholine action affects fiber permeability t K Even in atrial muscle, which is entirely free of vagal influence acetylcholine is formed by the choline acetylase system. Burns believes that this constantly formed acetylcholine effects a suitable resting membrane potential by affecting membrane permeability to K, and he has shown that atria that have ceased t beat have lost most of their ability to form acetylcholine.

If during acetylcholine perfusion, the atrial muscle is electrically stimulated, fibrillation occurs and will continue if the K concentration is simultaneously reduced in the bath Such fibrillation will persist unless (1) the acetylcholine perfusion is stopped or (2) the K concentration in the medium is raised Hoffman and Suckling[u] ha e shown that acetylcholine lengthens the period during which the fibers are excitable by shortening the repolarization process If electric stimulation can be thought of as leaving the fibers out of phase then the presence of acetylcholine pparently leads t fibers continuously and serially stimulating one another and thus fibrillating In such a situation, removal f cetylcholine by allowing the repolarization process t lengthen again makes continuous auto-re-excitation impossible

The detailed mode of operation of the well-known epinephrine-acetylcholine antagonism has been studied by Webb and Hollander[u] who have shown that epinephrine protracts atrial repolarization Of course, if this is true, then epinephrine should antagonize the action of acetylcholine in producing conditions under which fibrillation will occur Indeed, it has now been shown that electric stimulation that will produce fibrillation in the presence of acetylcholine is without such effect if adrenalin-acetylcholine mixtures are employed in the perfusate

It has been mentioned previously that fibrillation can be prod ced by electric stimulation during acetylcholine infusion only when the K concentration is reduced S ch fibrillation can be stopped either by removing acetylcholine or by raising the K concentration of the medium This lends credence to
iew that reduction in the length of the action potential by acetylcholine accompanied by an increased outward flux of K through membrane

more permeable Such a view has been strengthened further by the radioisotopic work of Harris and Hutter who have shown that a two- to threefold increase in the rate of loss of K^{42} from previously K^{42}-equilibrated preparations is occasioned by the addition of acetylcholine Their findings extend as well, to K^{42} uptake, although the magnitude of K^{42} inward flux is considerably less affected by acetylcholine than K^{42} outflux Their work also adds to the mounting evidence that the pacemaker region is electrochemically different from the peripheral atrial musculature in that acetylcholine affects the pacemaker area in its K^{42} permeability more than it does the rest of the atrium

Unfortunately there is little evidence bearing upon the question of whether membrane alteration brings about functional cardiac changes through specific effects on ion concentation differences, inside versus outside, or whether these effects are, instead mediated through rates of change of ion concentration or even possibly by rates of passage through the membrane in question. Burn[1] states that varying the rate of electric stimulation of the atria, in the absence of added acetylcholine, from frequency of 200/min to 1200/min increased the rate of K loss but little (0 76 mM/kg /min. at 200 to 0.94 mM/kg /min at 1200) *On the other hand* addition of acetylcholine increased the rate of K loss at all frequencies, the rate t 200 min being 1 36 mM/kg /min (or about double the rate seen in the absence of added acetylcholine) and rising to a value of 2 10 mM kg /min at a stimulation frequency of 800/min at which point fibrillation ensued Such findings tend to suggest that it is the *rate* of loss of K that is important and thus presumably but not necessarily is the rate of *net* loss

Effect f Foreign Inotropic Agents on Cardiac Ion Partition

Quinidine acts strongly as an antagonist to acetylcholine in cardiac muscle, as well as in smooth and skeletal muscle. In the atrium this material reduces both the amplitude and rate of contraction and, if the exposure to sufficient concentrations of quinidine is prolonged, atrial contractions cease If after quinidine depression, acetylcholine is added to such preparations, contraction begins again Acceptance of the mechanism of acetylcholine action as being that of increasing membrane permeability to K ions leads, then, to the assumption that quinidine acts by reducing K outflux Quinidine induced atrial arrest can be reversed by reducing the external K concentration. This procedure increases the inside-to-outside K gradient and such a stimulus to K efflux reverses quinidine action this fact tends to support the assumption that quinidine has opposite effects on membrane properties from those of acetylcholine. The analogy is not strictly true in that quinidine has no apparent effect on the resting potential of the membrane, whereas acetylcholine does.

Procaine and other surface anesthetics and diphenylhydramine may act in a manner similar to quinidine at least they all increase both the duration of the action potential and the duration of depolarization Digitalis, iodoacetate carbachol and physostigmine while producing no effects on the magnitude of either the resting potential or action potential, act to shorten action potential duration. Evidence from the work of Glynn[40] and Schatzmann,[41] who studied the action of strophanthin scillaren and other cardiac glycosides, indicates

that these substances probably exert their effects by altering either ionic partition or ionic transmembrane d namics

T nscapillary Ionic Movements

A discussion of ion mobility across what has the appearance of being a simpler membrane, the capillary endothelium, is pertinent. Although not much is known about it (it will be apparent that our ignorance of it is at least as great as our ignorance of the physiological effects of ions on the heart) it will illustrate the long way we have yet to go before ion effects are thoroughly understood

There is now a considerable history on the use of the isotopic tracer technique in revealing information concerning the disappearance of ions from the ascular system.[27 28] Although certain of the methods can be adapted to single tissues, most methods reported as utilized in the intact *animal reveal mean capillary* exchange rates, the results being an algebraic sum of many individual rates of exchange While methods such as that of Sheppard *et al*[28] produce data that mask a host of variables, they ha advantages in terms of simplicity and re producibility and in providing a large amount of pertinent ancillary data FIGURE 2 illustrates simultaneous time-concentration curves for a protein-bound dye (T 1824) Na and K^{42} following the sudden injection of a small slug of these materials int a large ein, the concentrations ha ing been determined in arterial blood obtained by rapid serial sampling From such curves t is possible to calculat (1) the cardiac output, (2) the modal cardiopulmonary circulation time, (3) the central blood olume, (4) the rate of loss of isotope from the vascular system, (5) the blood volume (6) the isotope space and, if mean blood pressure is available, (7) the total peripheral resistance.

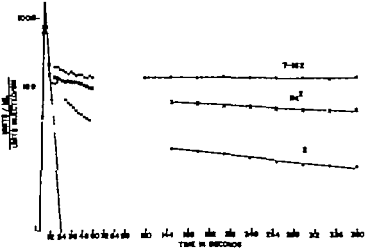


FIGURE 2 Time-concentration curves for T 1824, Na^{24} and K^{42} illustrating th extrapolat utilized in calculating cardiac output

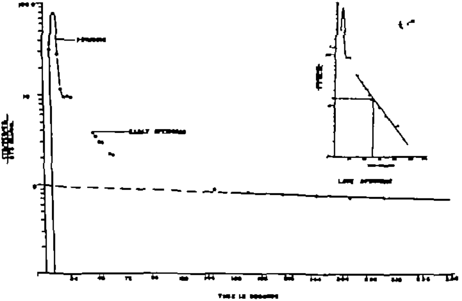


FIGURE 3. Typical K^{42} disappearance curve indicating the late aperiodic extrapolat to 0 time. The inserted diagram represents the semilogarithmic plot of the early aperiodic portion of the K^{42} disappearance curve corrected by the virtual concentrations derived from the extrapolat.

Inspection of these curves and of the type curve shown in FIGURE 3 indicates that, due to the heterogeneity of disappearance rates and the onset of ionic backflow from extravascular pools encountered in experiments with both rapidly or slowly exchanging isotopes, the curves do not remain linear and must be resolved into components. Slope analysis of the early aperiodic portion of the isotope curve (shown replotted in the insert of FIGURE 3) yields the mean rate of exchange of isotope between vascular and extravascular pools. For steady state conditions, this is equal to the outward transport rate expressed as the fraction of total Na or K that leaves the circulation per minute, or a value that Walker and Wilde[27] expressed as turnovers per minute.

Barlow *et al.*[28] have reported the results of such measurements in 65 normal dogs. These results indicate that Na and K leave the vascular system at individually constant rates, but at significantly different rates for the two ion species. Thus, the average vascular slope (transport rate) for Na^{24} is 1.38 ± 0.30 while that for K^{42} is 2.22 ± 0.39. The difference between these numbers is significant at the 0.1 per cent level. The disappearance rates for Na and K *are highly reproducible not only in the same animal, but also from one animal* to another and, indeed, from species to species. Furthermore, these rate constants appear to reflect fundamental properties of the endothelial barrier or the interaction of the ions with the barrier since severe alterations in hemodynamics *fail to alter the rates of loss*. Evidence has been presented by Sheppard *et al.*[29] that the individual tissue transport rates do not differ markedly from the mean rate. These authors showed that slopes of the early aperiodic portion of

isotope time-concentration curves derived from sampling different veins were quite similar even though such curves were variously displaced horizontally in time and vertically in concentration. These rates, then, are not circulation limited since it appears from experiments involving gross alterations in hemodynamics that as long as capillaries are available to the injected isotope the rate of loss is identical. It also appears that all capillaries have approximately the same properties as far as Na and K transfer is concerned.

Despite the difficulty encountered in changing the reported rate constants they can be changed in a number of ways that present interesting avenues of speculation regarding the functional anatomy of the endothelial barrier. Exposure of animals to whole-body X-radiation augments both Na and K transport rates. Intravenous histamine increases Na transport, but has no effect upon K. On the other hand, intravenously administered hyaluronidase increases K turnover rate about 60 per cent without altering Na transport. From the foregoing it is clear that Na and K normally leave the vascular system by routes or mechanisms that are not only different but that can be independently or mutually altered. Application of this method to labeled materials of different molecular sizes and configurations has also brought to light a number of interesting and challenging facts. The vascular slope (disappearance rate) of natural radioinsulin, for example, is about equal to the rate of loss of the Na ion. On the other hand, when such insulin is subjected to denaturation with alkali although there is but little change in molecular weight, the disappearance rate is very slow and approaches that of the protein-bound dye, T 1824.

Elucidation of the *modus operandi* of the capillary barrier, then, awaits the experimental description of the rates of loss of many other materials as well as the effects of other substances (such as aldosterone, cardiac glycosides, vitamins) and agencies (heat, cold, anoxia) upon these phenomena. Even then, only a beginning will have been made, for knowledge of the functional capacities of this and other membranes may explain much of new importance in diagnosis and therapeutics.

As an example of the combination of the rate-of-loss measurement with total tissue ion determination and calculations of tissue and plasma specific activities and relative specific activities in the elucidation of ionic transport and distribution characteristics, the recent work of Habib *et al.*[30] is useful. Following a two-hour period of vividialysis against a K-free fluid, the rate of loss of K from the blood of dogs was determined, as well as the electrolyte and water content of heart, liver and skeletal muscle. A significant increase in K content of cardiac muscle was found and liver Na was also significantly elevated. The mean rate of loss of K from the vascular system under these conditions (for example, 20 min after cessation of dialysis) was significantly reduced.

Such experiments generally raise more questions than they answer and yet they illustrate important points relative to ionic physiology. They point to the desirability of compiling data on the *dynamics* of materials transport; they remind us of our abysmal ignorance of ion partition; they illustrate the need for studies, not only of the steady state (if such exists) but of various phases of the numerous critical activities of body tissues; they beckon us with the

enticing possibility that the solution of these problems will bring a new era in the therapy for and prevention of disease

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EFFECT OF IONS ON THE ACTIVITY OF ENZYMES DERIVED FROM CARDIAC TISSUE

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Introduction

An analysis of the various factors involved in cardiac contractility must take into consideration the unique features of this highly irritable tissue, as well as those it holds in common with other more uniform types of muscle tissue. Such a constantly working muscle with its intrinsic as well as extrinsic nerve supply is peculiarly dependent upon its ionic environment, extracellular as well as intracellular. Alterations in the ionic composition of extracellular fluid often have profound effects. Thus, an elevation or depression of K^+ markedly alters the electrocardiogram and the heart beat. Since homeostatic control of the extracellular fluid composition is accomplished by many other tissues, kidney and liver in particular, the hypothesis has been advanced that many cardiac abnormalities do not stem from cardiac tissue dysfunction primarily but, secondarily, as a result of alteration in ionic composition of extracellular fluid.

The synergistic or, on the other hand, antagonistic functions of extracellular ions upon the irritable structures and membranes are discussed by other contributors to this monograph. Whether these ions exert their influence by effecting enzyme reactions at the surface membrane or merely alter the electric charge distribution of surface colloids is a matter of speculation. In unicellular organisms, yeasts, and bacteria, various ions can exert profound effects at the cell membrane.

Cardiac tissue, like most other tissues, controls its intracellular ionic composition for the most part by active transfer mechanisms, which are membrane phenomena in the first instance. These mechanisms have been extensively investigated in nerve, and some extension to muscle has been made. Thus, the virtual exclusion of Na^+ from the cell interior, even though the membrane is permeable to Na^+, is ascribed to an active Na^+ exclusion mechanism located in or near the membrane. The incorporation of inorganic phosphate, to which the membrane is not readily permeable, is an active process involving a specialized membrane transport mechanism. Even the uptake of glucose, which might be thought to enter the muscle cell by passive diffusion and then undergo enzymatic attack in the cytoplasm, seems to require a highly specialized, specific membrane mechanism. In the case of skeletal muscle this is the process considered by many workers to be controlled by insulin and other hormones.

Our knowledge of the enzymatic reactions underlying membrane transport phenomena is meager and is largely in the descriptive stage of development. Ion movements can be traced with radioactive elements, and the effect of varying such parameters as temperature, ion, substrate, or inhibitor concentrations has been described. The main difficulty is that mammalian cell membranes

are difficult to isolate and when apart from the intracellular environment they have few measurable enzymatic properties. Perhaps it is illusory to view the entire transport mechanism as occurring in the membrane the energy for the process is probably provided by the intracellular enzymatic machinery and communicated to the membrane.

The metabolic machinery of the heart is geared primarily to energy liberation By supplying appropriately labeled substrates it has been shown that the main pathways of metabolism are via the Emden-Meyerhof scheme of glycolysis, fatty acid degradation, and the citric acid cycle Hydrogen and electron transport to oxygen occurs on organized enzyme chains within the mitochondria. All of the cytoplasmic enzymes and subcellular particles are influenced by ions to a greater or lesser degree In a broad sense cellular metabolism carries out a dual function while it is providing the energy for the working muscle it is also maintaining the intracellular ionic environment, the high K^+ Mg^{++} $HPO_4^{=}$ and the low Na^+ and Ca^{++}

In most instances the only way to assess the effect of a given ion upon an enzymatic reaction is to study the reaction kinetics with as pure an enzyme as possible thus eliminating side effects that might cause spurious results This is not of course the situation in which the enzyme is found within the cell There a number of competing o supporting reactions may alter the activity of the enzyme Nevertheless, as a first approach results obtained on purified systems are at least guides to the characteristics of a reaction when it is integrated into a metabolic pattern From this point of view ion effects on the major enzymatic reactions concerned with energy release will be reviewed, and then certain generalizations will be discussed

Enzymes Concerned with Glycolysis

More is known about ion effects on this group than on any other primarily because so many of the enzymes have been isolated in a crystalline or highly purified state (TABLE 1) Heart tissue hexokinase as isolated from homogenates

TABLE 1
ION EFFECTS ON GLYCOLYTIC ENZYMES

Enzyme	Coenzyme and/or cosubstrate	*Activating ions*	*Inhibitory ions*
Hexokinase	ATP	Mg^{++}	Gluc 6-P
Phosphohexose isomerase	none	none	Co^{++} Mn^{++} Mg^{++}
Phosphofructokinase	ATP	Mg^{++} K^+ or NH_4^+	ATP Ag
Aldolase	none		Heavy metal
Triosephosphat dehydrogenase	DPN	Zn	Heavy metal
3-Phosphoglyceri kinase	ATP	Mg^{++}	
Phosphoglyceric mutase	2 3 di PGA	none	
Enolase	none	Mg^{++}	F^- Ca^{++} Sr^{++}
Pyruvic phosphoferase	ADP	Mg^{++} K^+ or NH_4^+	Ca^{++} Na Li
Lactic dehydrogenase	DPN	Zn	

Twenty to 30 per cent inhibition at 0 04 M

Adenosine triphosphat (ATP) adenosine diphosphate (ADP), diphosphopyridine nucleotide (DPN) 2 3 diphosphoglyceric acid (2,3 di PGA) Glucose 6-phosphat (Gluc-6-P)

exists in both a particulate and soluble form As with most of the kinases it is activated by Mg^{++} and the products of the reactions, ADP and glucose-6-phosphate, are *strongly inhibitory* Both the glucose and the phosphate moieties of glucose-6-phosphate are implicated in its inhibitory effect Phosphofructokinase also requires Mg^{++} and the partially purified brain enzyme is markedly stimulated by NH_4^+ although, unlike crude preparations, it is not stimulated by K^+ ion It is not inhibited by sulfhydryl blocking reagents such as iodoacetamide yet it is very sensitive to silver ions It must be emphasized that these observations pertain to the brain enzyme similar studies on enzyme from heart have not been made Aldolase resembles phosphofructokinase with respect to inhibitory agents heavy metal ions inactivate it while iodoacetate does not

The much studied triose phosphate dehydrogenase continues t reveal new facets of its structure It has recently been shown to contain zinc, and this metal may be one link between protein and the bound DPN that remains with the enzyme despite repeated recrystallization The other dehydrogenase in the glycolytic scheme lactic dehydrogenase is also a zinc-containing enzyme, and it likewise requires no other specific ion for its enzymatic activity

Enolase activation and inhibition are important because they illustrate one way in which both cations and anions influence these phenomena Mg^{++} the activator combines with the inhibitor F^- and phosphate to form a magnesium fluorophosphate complex that is the true inhibitor Although Mn^{++} and Zn^{++} in low concentrations are better activators than Mg^{++} for the yeast enzyme it is assumed that Mg^{++} is the preferred activator within the cell, since it is more effective at concentrations that are representative of intracellular concentrations.

Pyruvic phosphoferase is also of considerable historical importance because this was one of the first enzymes shown to require K^+ for maximum activity In fact the slight reversibility of this transphosphorylase reaction could not be demonstrated until the stimulatory role of K had been discovered. We now know of other reactions for synthesizing phosphoenolpyruvate that serve to circumvent the large energy barrier between it and pyruvate hence K^+ no longer plays such a vital role in reversal of glycolysis.

Enzymes of the Krebs Cycle

Ion requirements for this group of enzymes have not been thoroughly investigated (TABLE 2) When the enzymes are studied as part of the complex mitochondria of which they are integral parts, they are already in a medium optimal for preservation of mitochondria, generally containing appropriate concentrations of Mg^{++} Mn^{++} K and phosphate ions As they have been extracted and purified, more information about their requirements has appeared

Crystalline condensing enzyme, prepared by Ochoa *et al* from pig heart was always assayed with K^+ present In the assay employing phosphotransacetylase this was necessary since this enzyme requires K or NH_4 for activity however it left unsettled the ion requirements for the condensing reaction *In studying the same reaction* in an extract of frozen-thawed mito-

TABLE 4

ION EFFECTS ON ENZYMES THAT SUPPORT THE MAIN SCHEMES OF ENERGY METABOLISM

Enzyme	Coenzyme and/or cosubstrate	Activating ions	Inhibitory ions
Malic enzyme	TPN	Mn^{++}	malate
Oxalacetate decarboxylase		Mn^{++}	malonate^{-}
Pyruvic oxidase	DPN DPT CoA, lipoic		
Glutamic dehydrogenase	DPN or TPN	Zn	Ag Hg^{++}
Glutamic aspartic transaminase	Pyridoxal P	Mg	
Glutamic-alanine transaminase	Pyridoxal P		
Succinyl CoA transferase			

Pyridoxal phosphate (pyridoxal P) thioctic acid (lipoic)

order to maintain optimal activity of the Krebs cycle it is necessary to replenish various intermediates, particularly oxalacetate. The malic enzyme is present in heart tissue and may be the key enzyme for this purpose. It is polyfunctional, the same enzyme catalyzes the formation of malic acid from pyruvate and CO_2 and also the decarboxylation of oxalacetate which is subsequently formed from malate by the activity of malic dehydrogenase.[59] Manganese is required for both the CO_2-fixing reaction and the oxalacetate decarboxylase activity. Inhibition of the decarboxylase activity by malate would seem to be a useful protective device serving to maintain the oxalacetate for the vital condensation reaction with acetyl CoA.

The complex pyruvic oxidase reaction that is the main link between glycolysis and the Krebs cycle involves one or more enzymes and four cofactors. A preparation from pig heart was tested by measuring citrate formation in the presence of added condensing enzyme Mg^{++} and K^{+} ions.[61] When tested for its ability to form acetylphosphate in the presence of added bacterial transacetylase Mg^{++} was not required. From this it may be concluded that the oxidase does not require Mg^{++}. The requirement for other ions was not adequately examined.

Heart tissue contains considerable amounts of glutamic dehydrogenase an enzyme that might function to furnish α-ketoglutarate, hence another key substrate that serves to maintain the Krebs cycle. Vallee *et al.*[62] have shown that liver glutamic dehydrogenase is a zinc metalloenzyme, hence it is possible that the muscle enzyme also contains zinc. No other ion requirements have been reported other than NH_4^{+} which is a substrate in the reaction.

The transaminases have not been sufficiently purified to permit a rigorous analysis for trace metals, however it has recently been reported that Mg^{++} stimulates the activity of glutamic-oxalacetic transaminase as much as 80 per cent above the activity observed in its absence.[63]

Role of Ions in Enzymatic Reactions

A listing of ion requirements and other modifying effects of ions in enzymatic reactions serves the useful purpose of emphasizing those that must be present in order to assure adequate activity. In addition to considerations of speci-

ficity it would be desirable to delimit the concentrations of ions required for producing an optimal effect Furthermore it would be very desirable to know how a given ion works in a specific reaction or system, since this would be useful in investigations on the controlling mechanisms in enzymatic reactions Definitive explanations of ion effects in this area are few and, at the risk of reiteration, some representative theories will now be briefly examined.

It is useful to classify ions commonly involved in enzymatic reactions into the following categories (1) ions that are integral parts of an enzyme complex essentially nondissociable, and that remain with the enzyme through extensive purification procedures (2) ions that are bound to the protein moiety of an enzyme and often to an associated cofactor as well but are more or less dissociable and consequently must be present in the free state in some definite concentration (they may also be bound to cofactor or substrate in the first instance and then, secondarily to protein as a metal-substrate complex) and (3) ions that are bound feebly if at all, and serve mainly to provide a favorable environment for the enzyme

(1) *Ions as Integral Enzyme Components*

These complex enzymes have often been called the true metalloproteins Typical representatives contain iron as in the heme proteins and in certain flavoproteins In the former iron undergoes reversible oxidation reduction however it is uncertain whether the iron undergoes a valence change in reactions involving the flavoproteins Examples of enzymes containing copper are tyrosinase and also some recently discovered flavoproteins. Carbonic anhydrase is the classic zinc-containing enzyme more recently carboxypeptidase and a number of dehydrogenases have been characterized as zinc metalloproteins [36] Molybdenum-containing enzymes such as xanthine oxidase, and nitrate reductase also fall in this category

Except for certain heme proteins, our knowledge of the nature of the binding between metal and protein is meager Studies on yeast alcohol dehydrogenase and muscle lactic acid dehydrogenase have revealed the fact that there is an atom of zinc for every molecule of bound DPN which suggests that the metal is concerned with the binding of cofactor to protein Perhaps one of the best examples of the manner in which a metal might act as a binding agent and at the same time participate in the catalytic function of the enzyme comes from model studies with pyridoxal, the cofactor of the transaminases Baddiley[38] has shown that solutions containing of pyridoxal, pyridoxamine an amino acid a keto acid, and a metal such as Cu^{++} form mixed coordination compounds of the type illustrated in FIGURE 1 In the amino acid denoted by R has reacted with the pyridoxal part of the complex and the keto acid R with the pyridoxamine part This then undergoes electron and proton rearrangements as shown in *b* wherein R has become the keto acid and R the new amino acid Postulating that the metal could be coordinated to an enzyme this might represent the interconversion that occurs in an enzymatically catalyzed transamination Unfortunately the two most abundant transaminases have not been prepared in a state of sufficient purity to permit reliable assay

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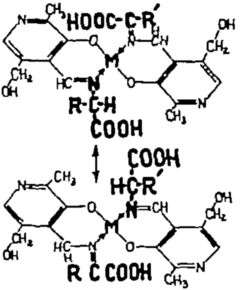


FIGURE 1

metals however a purified tyrosine-ketoglutarate transaminase from liver has been found t contain copper [26] With the recent improvement in glutamic aspartic transaminase purification,[27] it may be possible to examine the problem of metal requirement more critically

One may speculate that the chronic depletion of a metal required for the continual resynthesis of a metalloenzyme could lead to progressive diminution of active enzyme and that ultimately it might become the limiting step in energy release Indirect evidence that this can occur in a specific enzyme system has been presented by Vallee *et al* [28] whose studies on patients with postalcoholic cirrhosis led them to conclude that a conditioned zinc deficiency develops as a result of the recurrent bouts of alcoholism that lead to progressive diminution of liver alcohol dehydrogenase activity and, ultimately of protein metabolism

(2) Ions that Have a Cooperative Action

The ions most frequently encountered as activators in enzymatic reactions are Mg^{++} Mn^{++} and occasionally Ca^{++} A common property of all these is their ability to form chelate stru-tures with a variety of substrates, particularly those containing phosphate or condensed phosphate groups. Thus, Mg^{++} is an almost universal component of reactions involving *ATP* or *ADP* Manganese may also function in these reactions, frequently not as well as Mg^{++} On the other hand, Mn^{++} is much more efficacious in the decarboxylations of β-keto acids These ions play a dual role in chelating with the substrate they alter the electronic structure so as to make certain groups of the substrate more vulnerable to attack and at the same time, they furnish a link to the protein in forming the enzyme-substrate complex

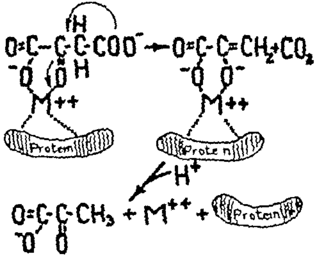


FIGURE 2

A most instructive example is again a model system, *the metal-catalyzed decarboxylation of dimethyloxalacetic acid investigated by* Steinberger and Westheimer[23]. If one projects their results to the enzyme-catalyzed decarboxylation of oxalacetic acid the mechanism shown in FIGURE 2 would be operative. The metal in this case is manganese. By chelating with the acid through the carboxyl and adjacent keto group manganese causes an electron shift from the carboxyl carbon, thus weakening the —C—COO bond and liberating CO_2. The resulting enol is then cleaved from the complex by a proton, and pyruvic acid is formed. By binding to the protein, the manganese chelate undergoes the reaction very rapidly. In the nonenzymatic model system, pyridine promoted the decarboxylation, thus giving additional support to the proposed mechanism with enzyme.

In the case of some Mg^{++}-stimulated phosphorylations notably those occurring in the fructokinase and phosphofructokinase reactions, Lardy and Parks[24] have pointed out that the ATP/Mg^{++} ratio is very important. If this ratio was increased to values greater than 1 progressively more inhibition was observed, presumably because the uncomplexed *ATP* interfered with the binding of Mg ATP to enzyme and thus diminished the rate of reaction. The exact mechanism of this effect is not known but again it would appear that the binding of Mg^{++} to the pyrophosphate group renders the terminal phosphate more reactive and likewise promotes the binding of *Mg ATP* to enzyme. Klotz[25] has discussed various types of protein binding with such complexes.

(3) *Ions that Facilitate the Union of Substrate Complex with Enzyme*

The monovalent cations, K^+ and NH_4^+, are the most common examples of this class. There are few reactions in which these ions are absolutely required. Only those catalyzed by pyruvic phosphoferase and, possibly,

condensing enzyme among those that we have been considering here. Several others are stimulated to various extents when these ions are present. The manner in which they exert their effect is especially puzzling since they do not seem to be bound to any important extent either to enzyme or substrate. However, Carr[80] employing an electrometric method has adduced evidence that K^+ is bound to a small extent with certain proteins. Although Na^+ was also bound, the extent of binding was usually smaller than with K^+.

The differences between K^+ and NH_4^+ on the one hand and Na^+ on the other have been emphasized over and over again. Quite generally when K^+ stimulates a reaction, the addition of Na^+ to the system will inhibit it. Yet this phenomenon is not a typical ion antagonism. Thus, Kachmar and Boyer[32] have shown that Na will stimulate the pyruvic phosphoferase reaction slightly even though it exerts a powerful inhibition on the K^+-stimulated reaction. From their kinetic data these authors exclude the binding of K^+ to substrate and suggest that it is in some sort of combination with enzyme, while Mg^{++}, the other indispensable ion in the reaction, is bound initially to substrate (pyrophosphate moiety of ADP).

The same preference for K^+ over Na^+ was well shown by Gamble[84] in his work with liver mitochondria. Carefully prepared mitochondria with their oxidative phosphorylating capacity intact will bind 25 times more K^+ than Na^+ per mg. N. This K^+ binding capacity is still retained although to a lesser extent in mitochondrial fragments prepared by digitonin treatment, which also preserves oxidative phosphorylation. However, mechanical fragmentation, which destroys the latter, also abolishes the K^+ binding ability.

It is difficult to visualize anything other than electrovalent bond formation between the monovalent alkali cations and proteins. It is known that polyacrylate macromolecules in solution can associate considerable amounts of alkali metal ion. Extension of this finding to proteins should also account for the high selectivity for K^+ or other cations. In FIGURE 3 is depicted a model to account for K^+ binding and the way in which this influences reaction velocity in an enzymatic system. Most of the enzymes we have been discussing bear a net negative charge at intracellular *p*H. This charge would tend to repel a highly negatively charged substrate such as one with a pyrophosphate group. Complexing of this group with Mg^{++} would reduce its electronegativity but not abolish it. The net negative charge on the protein is contributed by many carboxyl groups and, in some cases, by phosphate groups as well. If these occur within the protein structure (within the helix coils or elsewhere in the interior) a cation would have to penetrate to this site, and parameters such as hydrated size and mobility of the cation would influence the penetration. Association with these interior sites would reduce the net negative charge on the protein and permit a more facile approach of the substrate complex to the enzymatically active site. This might or might not be the same as the K[illegible]ttracting site.

The selectivity for [illegible]ations of certain dimensions brings to mind a class of compounds called clathrates[83, 86] formed by locking a "guest" compound within spaces formed by the union of several "host" compounds. There is considerable selectivity [illegible]f "guests"; if they are too large they cannot be enclosed

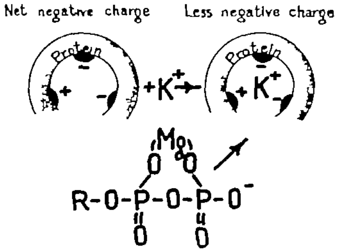


FIGURE 3

and, if too small, they can slip out of the cage like structure Zeolites, bentonites, and synthetic resins have been considered to be solid macromolecular structures of this type it would not be amiss to extend the analogy to proteins in solution.

In conclusion it should be emphasized again that even if we accurately define the ion requirements for an isolated enzyme, the ultimate goal must be to understand the factors that regulate its activity *in vivo* There is ample evidence that many enzymes cannot be working at maximum velocity within the cells Consequently even if the ionic environment is not optimum there may still be sufficient reserve enzyme activity to meet the ordinary needs. However one consequence of a chronic ion imbalance may be a progressive diminution of this reserve to a point where adequate function is impaired

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INTERACTIONS OF GLYCOLYSIS AND OXIDATIVE PATHWAYS

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When I was approached to participate in this symposium on metabolic factors in heart contractility with a review on glycolysis and the shunt pathway I attempted to convince the program committee that my ignorance of heart contractility is almost equalled by my ignorance of heart metabolism This did not eliminate me as a contributor to this monograph since I cannot speak about the carbohydrate metabolism of the heart, and since this is a symposium on the heart, I will discuss what I hope can be called the heart of carbohydrate metabolism

When we attempt to study the role of metabolic factors in the physiology of an organ such as the heart we try t learn as much as we can about the enzyme systems that function in that organ In order to do so, it is necessary to disrupt the organ structure and to isolate the individual enzymes Enzymology is a relatively young science To paraphrase Bernard Shaw "Youth is a wonderful thing it seems a pity to waste it on the purification of enzymes However isolation of the enzyme systems has been the most rational approach to the study of mechanisms and now that enzymology begins to approach maturity we find that some of the enzymologists are willing to part with some of the puritanical views of their scientifi adolescence

Previously enzymologists used intact tissue cells merely as starting material for the purification of enzymes The more brutally they could treat the tissues, the better they liked it. With the recognition of intracellular structures such as mitochondria and microsomes as metabolic organs, the enzymologists developed a more gentle approach t th cell Not only did this open a new world of multienzyme systems t them, but they ould now take advantage of the structural distribution of enzymes for purposes of purification

Among these enzymologists a few have taken a further step and turned to the study of enzymes in intact cells The difficulties that confront them here are quite different from those encountered with purified enzymes or even with ell-free multienzyme systems In order to evaluate observations that they make with intact cells, they must know th major alt rnative pathways they must learn what limits the rate of each of these multienzyme systems they must be aware of interactions between the majo systems and of the numerous side reactions that exist in the complex metabolic machinery of the cell Perhaps this generalization will become clearer when w deal with a more concrete problem—the tilization of glucose

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There are three major pathways in animal tissues responsible for the breakdown of glucose to CO_2 and water: (1) the glycolytic pathway, (2) the tricarboxylic acid cycle (TCA cycle) and (3) the oxidative pentose phosphate cycle (P-P cycle). These catabolic systems are interlinked with the metabolism of fats, proteins, and nucleic acids in an almost continuous network of enzyme-catalyzed reactions. These relations will not be discussed here. Instead, I shall concentrate on phenomena of interactions between the three major multienzyme systems of carbohydrate metabolism and point out some of their similarities and differences.

First I shall discuss the interplay between glycolysis and the TCA cycle. The enzymes of glycolysis and of the TCA cycle are structurally separated inside the cell and we can assume that there is little direct interaction at the enzyme level. However, both systems require nucleotide cofactors and inorganic phosphate. A phenomenon such as the Pasteur effect indicates that intracellular metabolism involves competitive processes. In our laboratory Wu has studied this phenomenon in recent years in intact ascites tumor cells, and we have also investigated it in a reconstructed system consisting of liver or heart mitochondria and a mixture of purified glycolytic enzymes. In the studies with this reconstructed system it was possible to vary at will the individual components, and I shall give a few examples of the competitive phenomena observed in such experiments.

It is now well established that carefully prepared mitochondria require ADP and inorganic phosphate for respiration. To permit the study of competitive phenomena, adenine nucleotides were used in catalytic amounts together with an ATP regenerating system. The effect of varying ATP concentrations in the presence of excess inorganic phosphate, hexokinase, and glucose is shown in FIGURE 1. In most subsequent experiments, 3 to 5 × 10^{-4} M ATP were used, which is just below optimal. Instead of glucose and hexokinase, ATPase was added in some experiments, as shown in FIGURE 2. We can see that a pronounced stimulation of respiration was produced by the addition of this enzyme. A comparison of FIGURE 1 and FIGURE 2 shows that, while there was hardly any respiration without added adenine nucleotide even in the presence of glucose and hexokinase, considerable respiration was obtained when catalytic amounts of ATP were added alone without an acceptor system. In fact, FIGURE 2 is not entirely representative in this respect as, in most experiments, only two- to threefold stimulation was achieved by the addition of hexokinase or apyrase.

We suspected that respiration in the presence of catalytic amounts of ATP might be due to mitochondrial ATPase activity. Such an activity was readily demonstrated in our mitochondrial preparations. I should say "readily" because other investigators have reported difficulties with accurate measurements

Abbreviations used throughout this paper: ATP and ADP for adenosine triphosphate and adenosine diphosphate; DPN and TPN for oxidized diphosphopyridine nucleotide and triphosphopyridine nucleotide; G-3-p for glyceraldehyde 3-phosphate; E-4-p for erythrose 4-phosphate; G-6-p for glucose 6-phosphate; F-6-p for fructose 6-phosphate; S-7-p for sedoheptulose 7-phosphate; 6-PGA for 6-phosphogluconate; FDP for fructose 1,6-diphosphate; SDP for sedoheptulose 1,7-diphosphate; PEP for phosphoenol pyruvate; EDTA for ethylenediamine tetraacetate; P-P cycle for pentose phosphate cycle; TCA cycle for tricarboxylic acid cycle.

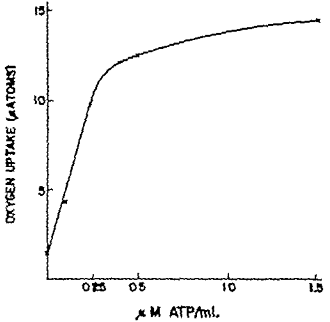


FIGURE 1 Effect of ATP concentration on respiration of mitochondria Each Warburg flask contained in a final volume of 2 ml 60 μmoles of DL-histidine 20 μmoles of K glutamate, 50 μmoles of K phosphate 40 μmoles of KCl 50 μmoles of glucose, 10 μmoles of $MgCl_2$ 3 6 units of hexokinase 4 3 mg of rat liver mitochondria Final pH 7 4 The vessels were shaken in a Warburg respirometer for 60 min at 30° C

of mitochondrial ATPase and we too have encountered them. We therefore developed a new assay procedure for ATPase that not only has the advantage that it can be carried out at more physiological levels of ATP but that it is also more reproducible The assay contains an ATP regenerating system consisting of phosphoenol pyruvate magnesium, an excess of pyruvate kinase, and catalytic amounts of ATP Inorganic phosphate formation was found to be proportional to the amount of mitochondria or apyrase added Furthermore mitochondrial ATPase activity measured by this procedure was found to be several times higher than when measured by the conventional method without ATP regeneration, as shown in TABLE 1 The most plausible reason for this discrepancy in the two assays was that ATPase is inhibited by ADP In this rare instance an obvious explanation turned out to be a correct one. ADP at concentrations beyond 10^{-4} M was found to inhibit mitochondrial ATPase an observation made previously by Kielley and Kielley The inhibition of ATPase by ADP may well represent a regulatory mechanism for the curious and apparently wasteful ATPase activity of mitochondria.

Just as the addition of a phosphate acceptor system enhances respiration of mitochondria, the addition of a competing phosphorylating system, such as ATP-creatine transphosphorylase (FIGURE 3) or glycolysis (TABLE 2) was found to inhibit respiration The inhibition was reversed by addition of excess ADP or 2 4-dinitrophenol, which indicated the operation of a competitive mech-

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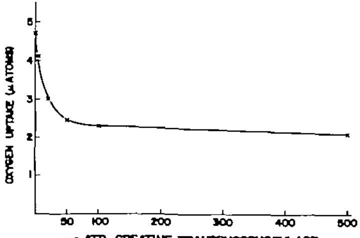


FIGURE 3 Inhibition of mitochondrial respiration by the phosphocreatine creatine kinase system Each Warburg flask contained in final volume of 1 ml 25 μmoles of K-phosphate, 30 μmoles of histidine 10 μmoles of glutamate 20 μmoles of fructose 20 μmoles of KCl, 0 5 mg of DPN 5 μmoles of $MgCl_2$ 0 33 μmoles of ATP 10 mg of phosphocreatine 0 6 units of hexokinase 4 8 mg of rat liver mitochondria suspended in 0 1 ml of 0 25 M mannitol containing 1 mg /ml neutral EDTA and ATP creatine transphosphorylase (creatine kinase) as indicated above The final pH as 7 4 The vessels ere shaken for 30 min at 27° C

TABLE 2
INHIBITION OF RESPIRATION GLYCOLYSIS
(CRABTREE EFFECT)

Additions to mitochondria	Oxygen uptake	Lactic acid	Pi esterified
	μatoms	μmoles	μmoles
—	3 1	—	2
Hexokinase	5 6	—	11
Hexokinase + glycolytic enzymes	3 4	11	14

T final volume of 2 ml ere added 20 μmoles of K glutamate, 50 μmoles of K-phosphate, 60 μmoles of histidine 40 μmoles of KCl 50 μmoles of glucose 10 μmoles of $MgCl_2$ 1 μmole of ATP 1 mg of DPN 6 mg of rat liver mitochondria, 700 μg of glucose-3 phosphat dehydrogenase 1 2 units of hexokinase and 1 2 units f phosphofructokinase In this experiment 2 mg of crude fraction from rabbit muscle served as the source of glycolytic enzymes The vessels are shaken for 30 min at 30° C

It was also possible to imitate the Pasteur effect in these reconstructed multienzyme systems As shown in TABLE 3 the formation of lactic acid was markedly inhibited when respiring mitochondria were added t a glycolyzing system. This experiment was carried out with limiting amounts of adenine nucleotides and excess of inorganic phosphat In order to perform similar experiments in the presence f limiting amounts of inorganic phosphate t was necessary t devise a system of glycolysis that proceeds under such conditions As sho in FIGURE 4 glycolysis can be maintained t low Pi concentrations by sel th correct amount of ATPase. Without ATPase little lactic acid was

TABLE 3

PASTEUR EFFECT IN A RECONSTRUCTED SYSTEM

	Oxygen uptake	Lactic acid
	μatoms	μmoles
Glycolytic system, no mitochondria	—	8.9
Glycolytic system with mitochondria	5.9	5.6

Average of 5 experiments

Meyerhof oxidation quotient = 3.3

Each Warburg vessel contained in a final volume of 1 ml.: 30 μmoles of histidine; 10 μmoles of K glutamate; 25 μmoles of K phosphate; 20 μmoles of KCl; 20 μmoles of fructose; 0.5 mg. of DPN; 0.3 μmole of ATP; 5 μmoles of $MgCl_2$; 0.25 units of hexokinase (assayed with glucose as substrate); 0.6 units of phosphofructokinase; 3.2 units of aldolase; 4.8 units of glyceraldehyde-3-phosphate dehydrogenase; 12.5 unit of phosphoglycerate kinase; 2.7 units of phosphoglycerate mutase; 8.8 unit of enolase; 3.8 unit of pyruvate kinase; 8.7 units of lactic dehydrogenase; and 0.05 μmoles of 2,3-diphosphoglycerate. The vessel, which was incubated in the presence of mitochondria, contained 7.2 mg. mitochondrial protein suspended in 0.1 ml. of 0.25 M mannitol containing 1 mg./ml. of neutral EDTA. The final pH was 7.4. The vessels were incubated for 30 min. at 27° C. and deproteinized by the addition of 1 ml. of 10 per cent TCA.

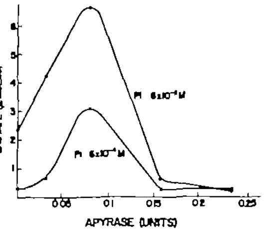


FIGURE 4. Effect of apyrase on glycolysis. Each test tube contained in a final volume of 0.5 ml.: 15 μmoles of DL-histidine; 0.25 μmoles of K fumarate; 0.25 mg. of DPN; 12 μmoles of fructose; 10 μmoles of KCl; 0.15 μmoles of ATP; 2.5 μmoles of $MgCl_2$; 0.3 or 3 μmoles of phosphate; 0.12 units of hexokinase (assayed with glucose as substrate); 0.3 units of phosphofructokinase; 2.4 units of aldolase; 2.4 units of glyceraldehyde-3-phosphate dehydrogenase; 4 units of phosphoglycerate kinase; 2 unit of phosphoglycerate mutase; 2 units of enolase; 1.85 units of pyruvate kinase; 4 units of lactic dehydrogenase; 50 μg. of myokinase; 0.025 μmoles of 2,3-diphosphoglycerate; and apyrase in varying amounts as shown in the figure. The final pH was 7.4. The mixtures were incubated for 30 min. at 27° C. and deproteinized by the addition of 0.3 ml. of 10 per cent TCA.

as expected in view of the need for inorganic phosphate for glycolysis while excess of ATPase inhibited glycolysis because it effectively competed with hexokinase for ATP.

Because of the rather narrow range of ATPase activity optimal for glycolysis, the experiments on the Pasteur effect in the reconstructed system with limiting

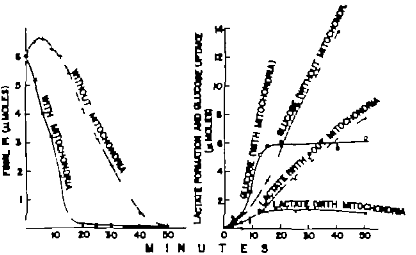


FIGURE 5. Kinetics of the glycolytic system at low adenine nucleotides and phosphate in the presence and absence of mitochondria. The flasks contained each in a final volume of 5.5 ml: 165 μmoles of DL histidine, 110 μmoles of K glutamate, 2.75 mg of DPN, 1.65 μmoles of ATP, 110 μmoles of KCl, 83 μmoles of glucose, 33 μmoles of K phosphate, 27.5 μmoles of $MgCl_2$, 2.6 units of hexokinase, 4.4 units of phosphohexose isomerase, 3.3 units of phosphofructokinase, 26 units of aldolase, 26 units of glyceraldehyde-3-phosphate dehydrogenase, 52 units of phosphoglycerate kinase, 23 units of phosphoglycerate mutase, 21 units of enolase, 20 units of pyruvate kinase, 44 units of lactic dehydrogenase, 550 μg of myokinase, 0.61 units of apyrase, 0.275 μmoles of 2,3-diphosphoglycerate, and 0.55 ml of 0.25 M mannitol containing 1 mg/ml of neutral EDTA or 97.5 mg mitochondrial protein suspended in the same medium. The final pH was 7.4. The flasks were shaken at 28° C and at the times specified in the figure, 0.2 ml of the reaction mixtures were pipetted into (1) 1 ml of 10 per cent TCA and (2) 1 ml of 0.3 N $Ba(OH)_2$; then 1 ml of 0.3 ml $ZnSO_4$ was added. The values are reported for 1 ml of incubation mixture and were corrected for evaporation.

inorganic phosphate concentrations were more difficult to execute, and it was necessary to follow glycolysis at various time intervals as shown in FIGURE 5. It can be seen from the right-hand portion of FIGURE 5 how the presence of mitochondria inhibits both lactate formation and glucose uptake; from the left-hand portion of the FIGURE 5 one can deduce the reason for this inhibition. In the presence of mitochondria, inorganic phosphate is depleted so rapidly that it is not available for either glycolytic or oxidative regeneration of ATP.

It must be understood that we look upon these experiments with the reconstructed system only as models for the study of interaction of multienzyme systems. Although we have produced a Crabtree or a Pasteur effect with these enzymes, we cannot state that these experiments are representative of intracellular events since we do not know what is limiting in the intact cell and since competitive effects can be obtained in the cell-free system by either limiting Pi or ADP or both. However, the above experiments enabled us to understand under what conditions competitive phenomena take place and prepared us for the next step, the investigation of the intact cells. Before turning to this aspect, however, I shall discuss some of the interactions between the glycolytic pathway and the pentose phosphate cycle.

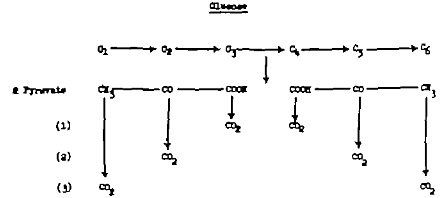


FIGURE 6 Sequence of carbon oxidation of glucose during TCA cycle

The glucose molecule which travels via the Emden-Meyerhof pathway is split into 2 molecules of pyruvate in such a manner that carbons 3 and 4 of glucose become the carboxyl group of pyruvate During the subsequent oxidation via the Krebs cycle the carboxyl group of pyruvate is detached first, then carbon 2 is oxidized, and finally the methyl carbon of pyruvate is oxidized t CO_2 With reference to the goucose molecule the sequence of oxidation is carbons 3 and 4 first, carbons 2 and 5 second and carbons 1 and 6 last (FIGURE 6)

In the course of the pentose phosphate cycle shown in FIGURE 7 arbon 1 is oxidized first ribulose-5-phosphate, the product of oxidation cycles back to glucose-6-phosphate Carbon 2 of the original glucose is now carbon 1 of the newly formed glucose-6-phosphate and again is oxidized to ribulose-5-phosphate. After another recycling of the pentose phosphate, carbon 3 is oxidized in the same manner after becoming carbon 1 of the newly formed glucose-6-phosphate What remains of the original glucose is a molecule of glyceraldehyde 3-phosphate which is converted to dihydroxyacetone phosphate and condensed with another triose phosphate to FDP FDP is cleaved to fructose-6-phosphate which re-enters the cycle and thus carbons 4 5 and 6 are burned by the same mechanism.

We can see, therefore that the sequence of carbon oxidation differs, depending on which of the two alternative pathways of glucose oxidation functions. If we attempt to evaluate the relative contributions of these pathways by using C_1- or C_6-labeled glucose we must realize that we are dealing with rate differences in complex multienzyme systems In cells with very active transketolase and transaldolase there can occur such a rapid scrambling of the carbons of glucose that it becomes difficult to evaluate pathways by the use of glucose labeled in a single carbon [4] Fortunately in most mammalian tissues the P P cycle is slow enough to permit qualitative studies of the mode of glucose utilization by intact cells but in view of the exchange reactions catalyzed by transketolase and transaldolase it seems almost impossible to arrive at accurate quantitative conclusions

Let us consider the kind of interactions between glycolysis and the P P cycle

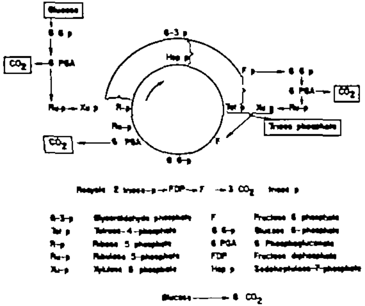


FIGURE 7 The oxidative pentose phosphate cycle.

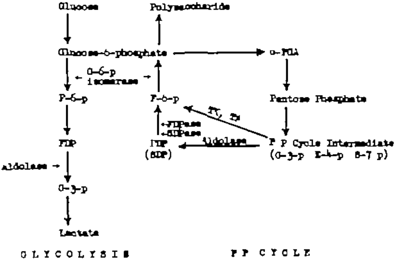


FIGURE 8 Interrelation between glycolysis and the P P cycle

that we might expect FIGURE 8 illustrates some of the synergisms and antagonisms between the glycolytic and the pentose phosphate pathways. Hexokinase acts in both systems in the same direction namely from glucose to glucose-6-phosphate. However in the case of three other enzymes, gluco-

phosphate isomerase aldolase and glyceraldehyde-3-phosphate isomerase the reactions proceed in glycolysis in the direction of lactate formation in the P P cycle the reactions proceed in the opposite direction namely toward glucose-6-phosphate formation At these points there is an interlocking of the metabolic wheels that forces them to work in opposite directions Indeed if the P P cycle is very active one can visualize it gears pushing the triose phosphate formed by glycolysis back to glucose-6-phosphate and, perhaps, even farther toward polysaccharide synthesis This reversal can proceed via FDPase as shown in FIGURE 8 or via another sequence written as follows

(1) G-3-p + S-7-p → E-4-p + F-6-p (transaldolase)
(2) DHA-p + E-4-p → SDP (aldolase)
(3) SDP → S-7 p + Pi (SDPase)

Sum 2 triose-p → F-6-p + Pi

The enzyme that catalyzes reaction 3 which we refer to as SDPase has been discovered quite recently It appears to be widely distributed in microorganisms, plants, and various animal tissues An SDPase purified from yeast was found to have a high degree of specificity The enzyme cleaves SDP to S-7-p and Pi it does not attack S-7 p F-6-p or FDP In contrast to an FDPase from spinach leaves that acts only at an alkaline pH, SDPase is active at pH 7 0 and may therefore play an important role in the reversal of glycolysis

To recapitulate we can now see how the pentose phosphate cycle may participate in reversing reactions of glucose catabolism. Transketolase and transaldolase push the pentose phosphates back to fructose-6-phosphate the sequential action of transaldolase aldolase and SDPase together push erythrose-4-phosphate and triose phosphate back to the hexose level

The oxidation of glucose via the TCA cycle and via the PP cycle have two factors in common Mg^{++} and thiamine pyrophosphate which are required for the oxidation of the α-keto acids in the Krebs cycle and for transketolase action. On the other hand there are some striking dissimilarities between these two multienzyme systems DPN is required for both glycolysis and oxidative phosphorylation TPN is required for the PP cycle Inorganic phosphate is an essential reactant in glycolysis and oxidative phosphorylation, but it inhibits the direct oxidation of glucose-6-phosphate via the shunt pathway [7]

Space permits only a very cursory review of our attempts to study intact ascites tumor cells Eling Kvamme began these investigations of the Crabtree effect in ascites tumor cells several years ago in our laboratory As circumstances did not permit him to complete the work for publication, I shall describe the experiments he carried out on the effect of glucose concentrations on the relative participation of alternative pathways of glucose breakdown Glucose labeled in C or C_6 position was added to ascites tumor cells at high and low concentrations of glucose both in the presence and in the absence of 2 4-dinitrophenol TABLE 4 shows that at high glucose concentrations the oxidation of C_6 which represents the TCA cycle, is inhibited while C_1 oxidation, which represents the first step of the PP cycle is favored The C_1/C ratio changes from 2 at low glucose concentrations to a ratio of 8 at high glucose concentrations. In the second experiment, TABLE 5 the effect of dinitrophenol

TABLE 4

EFFECT OF GLUCOSE CONCENTRATION ON OXIDATION OF CARBON 1 AND 6 OF GLUCOSE BY ASCITES TUMOR CELLS

Substrate	Total $C^{14}O_2$ ct /min	
	Low glucose (1 × [illegible] M)	High glucose (1 × [illegible] M)
C^{14} 1 glucose	12 800	23 400
C^{14} 6-glucose	6 050	2 900

Ascites tumor cells (30 mg protein) suspended in sodium chloride phosphate buffer solution at pH 7 4 (containing 103 μmoles of NaCl and 33 μmoles of Pi per ml) were shaken in Warburg respirometer for 30 min at 35° C in the presence of radioactive glucose, as indicated above $C^{14}O_2$ was collected in 0 2 ml of 10 N KOH in the center cup after tipping 0 2 ml of 10 N H_2SO_4 from the side arm at the end of the incubation period The specific activity of C^{14} 1 glucose used in both experiments was 36,000 ct /μmole/min C^{14}-6-glucose was 58 000 ct /μmole/min

TABLE 5

EFFECT OF 2 4-DINITROPHENOL ON OXIDATION OF CARBON 1 AND 6 OF GLUCOSE BY ASCITES TUMOR CELLS

Substrate	Total $C^{14}O_2$ ct /min	
	Low glucose	High glucose
C^{14} 1 glucose	4000	8120
C^{14} 1 glucose + DNP (10^{-4} M)	4800	9400
C^{14} 6 glucose	3700	2100
C^{14} 6-glucose + DNP (10^{-4} M)	4000	4500

Experimental conditions as in TABLE 3

is shown. Dinitrophenol has little effect on the oxidation of C but eliminates the inhibition of C_6 oxidation

More recently Wu made extensive studies on the limiting factor in the carbohydrate metabolism of ascites tumor cells To summarize his findings

(1) Enzymes of glycolysis are present in large excess and do not limit glucose utilization This was clearly revealed by an analysis of the individual enzymes of glycolysis, as well as by measuring glycolysis in fortified homogenates, which was found to be several times higher than that of the intact tumor cells

(2) Determinations of intracellular concentrations of adenine nucleotides, inorganic phosphate, and intermediates of glycolysis strongly pointed to inorganic phosphate as limiting factor Since ATP levels were high during oxidation, it was concluded that, either because of compartmentalization or because of the presence of inhibitor the utilization of ATP for glucose phosphorylation was inefficient under these conditions

(3) The limitation of glycolysis by the intracellular Pi concentration was also indicated by experiments on the effect of high concentrations of added inorganic phosphate It was found that, both aerobically and anaerobically high concentrations of inorganic phosphate stimulate glucose uptake and lactic acid

formation with the result that the per cent Pasteur effect diminishes with increasing phosphate concentrations.

(4) The transport of inorganic phosphate, therefore apparently limits carbohydrate metabolism of ascites tumor cells. As determined by phosphate analysis, as well as by P^{32} measurement the transport of inorganic phosphate is promoted by either glycolysis or respiration These two metabolic processes can be used independently since their contributions to Pi uptake are almost additive

In studying the carbohydrate metabolism of intact cells we have begun with a study of enzymes we proceeded to a study of the intracellular concentrations of intermediates and cofactors and we are still left with an unsolved problem of permeability and active transport

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DISEASES OF GLYCOGEN STORAGE WITH SPECIAL REFERENCE TO THE CARDIAC TYPE OF GENERALIZED GLYCOGENOSIS

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Diseases of glycogen storage are rare disorders due to congenital and at times familial errors of carbohydrate metabolism leading to abnormal accumulation of glycogen in certain organs of the body which as a consequence may become greatly enlarged. In most instances several organs or tissues are affected to some extent, but the largest accumulations of glycogen are apt to be found either in the liver or in the heart, so that two distinct subdivisions of glycogen disease have been traditionally distinguished: the hepatic form and the cardiac form. The latter is in reality a generalized glycogenosis whose major clinical expression is cardiac enlargement and failure of the heart function. In addition, other types of disturbances leading to glycogen storage have been recognized in recent times.

In the last five years momentous advances have been made in the study of these diseases. These developments have shed an entirely new light on the study and diagnosis of these conditions and have clarified to a certain extent the true nature of some of them. The chemical studies of Gerty Cori[4] in particular have greatly clarified the subject and have shown that in some instances a single enzyme defect is responsible for the disturbances in carbohydrate metabolism and for the abnormal accumulation of glycogen.

Chemical Background

Glycogen is a highly branched polysaccharide with a treelike structure. Glucose is its only building block. Most glucose units are joined together by α-1,4-glucosidic linkages to form chains. In addition there are α-1,6-linkages at branch points (FIGURE 1).

The enzyme phosphorylase only makes or breaks the 1,4-linkages. It synthesizes straight-chain polysaccharides. When phosphorylase acts in conjunction with amylo-1,4→1,6-transglucosidase or branching enzyme the product in the human is glycogen. For the splitting of 1,6-bonds still another enzyme, amylo-1,6-glucosidase or debranching enzyme is required. When glycogen is degraded by phosphorylase the chain loses one glucose residue and glucose-1-phosphate is formed. Phosphorolysis stops as the outermost tier of branch points of each molecule is approached since phosphorylase can neither break nor bypass the branch points. The polysaccharide thus obtained is called phosphorylase limit dextrin.

The debranching enzyme then removes free glucose from this limit dextrin. Glucose and limit dextrin minus the outermost tier of branch points are the reaction products. Phosphorylase in the presence of inorganic phosphate can

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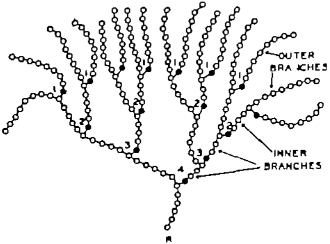


FIGURE 1. Model of segment of glycogen molecule. Open circles, glucose residues in α-1,4 linkage; black circles, residues in α-1,6 linkages. Reproduced by permission of Academic Press.

act again on this polysaccharide. Acting one after the other, the two enzymes degrade the entire glycogen molecule. In the digestion mixture only glucose and glucose 1-phosphate are found. Their ratio is a measure of the percentage of branch points in each glycogen sample. Therefore the separate and alternating use of phosphorylase and debrancher is an enzymatic method for the study of glycogen structure.

In body tissues glucose 1-phosphate is transformed to glucose-6-phosphate, a reversible reaction mediated by the enzyme phosphoglucomutase.

The liver as an organ is entrusted with the highly important task of maintaining blood glucose at normal levels. It performs glycogenolysis, essential to proper homeostasis, by breaking down glycogen through, of course, glucose-6-phosphate to glucose, which is then made available to the blood stream. This last reaction is mediated by a specific enzyme, the hepatic glucose-6-phosphatase, which is also found in small amounts in the kidneys. The function of glycogenesis, of replenishing the glycogen stores and of decreasing a raised blood level of glucose, is carried out by phosphyorylating glucose to glucose-6-phosphate, a reaction mediated by the enzyme hexokinase. It can then be transformed to glycogen through the reactions described in the preceding paragraph.

Aside from this highly specific function of the liver, glucose, in itself a metabolically inert substance, is phosphorylated in all normal cells to glucose-6-phosphate through the catalytic action of hexokinase, an enzyme ubiquitously present in all tissues. From this point it may go one of two ways, depending on the needs of the body at the moment. It may be transformed to glycogen and stored for future use, or it may be broken down by way of the Emden-Meyerhof cycle to pyruvate. Several pathways are open for further processing of pyruvic acid, again depending on the requirements of the cells at the time.

Recognized Types of Diseases of Glycogen Storage

Von Gierke[4] in 1929 described the clinical and pathological findings in two children who had died with massive enlargement of the liver and kidneys due to deposition of glycogen in these organs He named the condition hepatonephromegalia glycogenica Chemical studies were subsequently carried out by Schoenheimer[6] on material from one of these patients, showing that the glycogen from this case could not be degraded by minced normal human liver

In 1932 Pompe[7] in Holland and Bischoff[8] and Putschar[9] in Germany independently described patients dying in infancy with an enormous enlargement of the heart du to diffuse deposition of glycogen in this organ. The liver and kidneys were not significantly affected in these infants. By analogy with the cases of von Gierke s syndrome, Pompe named this ondition cardiomegalia glycogenica It was recognized from the beginning that there was no similarity in the clinical picture between what came to be called the hepatic and the cardiac types of glycogen storage disease It was thought for many years, however that the metabolic defect causing the excessive accumulation of glycogen was similar in both types

The confusion has been heightened since by the reporting of cases charact ized by deposition of glycogen in various organs or various combinations of organs. These reports have been poorly documented and they have relied primarily on clinical and pathological findings. It must be stressed that m rphologic changes due to the accumulation of glycogen are very similar even though this polysaccharide may be quite different in its structure With few exceptions, either there were no chemical studies or they were limited to determination of the percentage of glycogen present in the tissues subjected t analysis as compared w th normal findings In very few instances the behavior of glycogen in the presence of healthy minced liver tissue was investigated

In 1950 two cases of the cardiac type of glycogen storage disease were reported from this clinic[1] and extensively studied from the clinical pathological and chemical standpoints From the findings in these patients and a review of the literature[2] it was concluded that the cardiac and hepatic types of glycogenosis were entirely separat diseases attributable to different metabolic errors It was suggested by Stetten and reported in our paper[1] that the length of the outer branches of glycogen might be abnormal This hypothesis proved to be prophetic in view f subsequent developments. However it is only in recent years and with the application of the techniques developed by Cori[4] that some light has been shed on this obscure field

To put the cardiac type of generalized glycogenosis in the proper perspective the various types of disease of glycogen storage must first be briefly considered Five distinct types have been recognized thus far (TABLE 1) Types I, II, III and IV are familial The information availabl on type V is inadequat

Type I This type the most ommon, is the so-called hepatic glycogen storage diseas the hesic disease of von Gierke of which more than one hundred cases ha e been reported many more ha been seen without being registered in the literature In this form th liver and at times, the kidneys are primarily affected and usually greatly enlarged. The chemical structure of the

TABLE 1
DISEASES OF GLYCOGEN STORAGE

Type	Organs affected	Cases reported	Glycogen structure	Enzyme missing	Glycemic response to epinephrine
I Hepatic type (von Gierke's disease)	Liver kidney	>100	Normal	Absent Glucose 6-phosphatase	Absent
II Cardiac type (generalized glycogenosis)	All tissues	>20	Normal	?	Normal
III Limit dextrinosis (Cori)	Liver muscle, heart	8	Abnormal short outer branches	Absent Debranching enzyme amylo 1 6-glucosidase	Normal
IV Liver (cirrhosis)	Liver retic endoth syst	1	Abnormal long outer branches	Absent? Branching enzyme amylo-1 4 → 1 6 transglucosidase	Normal
V Muscle type	Muscle, others?	1	Normal	?	Normal

glycogen is normal, but the glucose-6-phosphatase in the liver and sometimes in the kidneys is absent or reduced. As a consequence the carbohydrate cycle in the liver is interrupted and glycogenolysis does not take place in the usual manner Clinically hypoglycemia, ketosis, and hyperlipemia occur and there is failure of the normal glycemic response after injection of epinephrine This latter is a very important clinical test for this type of disease.

Type II The cardiac type of generalized glycogenosis or Pompe s disease is much less common only about twenty cases that are acceptable as such have been described so far Here again the structure of glycogen is normal, and the defect leading to the massive accumulation of glycogen throughout the body and having its most important clinical expression in the cardiomegaly is unknown All clinical investigations of carbohydrate function and specifically the epinephrine tolerance test are negative as the glycogenolytic function of the liver is preserved This particular form will be further discussed later in this paper

Type III This form is characterized by a glycogen of abnormal structure with short outer branches due to absence of amylo-1 6-glucosidase activity (debranching enzyme)[11] leading to a glycogen molecule resembling a "limit dextrin Cori, who first discovered this type of glycogenosis, has proposed the name limit dextrinosis, but perhaps Cori s disease might be a simpler one Specimens from nine cases have been analyzed by Cori thus far [12] but only one of them has been reported in the clinical literature [13] Available information is therefore scanty It is apparently established that this condition is familial,[12] that the liver is greatly enlarged, and that muscle weakness of moderate degree may be present Glycemic response to injection of epinephrine occurs, but may be less than normal The heart is apparently not enlarged roentgenographically or electrocardiographically although this is not known for all cases The pathological findings at autopsy also are not available although, on chem-

ical analyses, specimens from two patients who had post mortem examinations[11] showed glycogen in excessive amounts in the myocardium, as well as in the liver and in the striate muscle The percentage of this polysaccharide in the myocardium was lower than in the cardiac type of glycogen storage disease Cori records that in som instances a diagnosis of the hepatic type f glycogenosis (von Gierke s disease) had been made clinically but chemical analysis showed the glycogen to be abnormal and the case in reality t belong in this group

Type IV The liver and reticuloendothelial systems are affected in this form of the disease and there is marked hepatic cirrhosis and hepatomegaly Only *one case, observed at Babies Hosp tal, New York, N Y has been reported* in which chemical studies were done[4, 14] The structure of the glycogen was abnormal in that this polysaccharide had few and excessively long outer branches it was thought to be due to decreased activity of the branching enzyme amylo-1 4-1 6-transglucosidase

Type V Even less information exists concerning Type V[15] One patient, also seen at Babies Hospital, clinically presented as a child two and one half years old with general muscle weakness and palatal paralysis Cases similar to this one have been described in the literature, but not studied chemically Striat muscle on pathological examination had an appearance similar to that of types II and III. The heart was uninvolved clinically roentgenographically and electrocardiographically and tests of carbohydrate function were nor mal Chemical examination of a biopsy specimen of striate muscle revealed that the glycogen content was 13 per cent, but that the structure of the glycogen was normal.

It is worth while to note again that the pathological appearance may be similar in different types of glycogen storage disease and that only chemical analysis, in addition to the clinical findings, has given a clue as t the basic process

Cardiac Glycogen Storage Disease

At Babies Hosp tal three cases of generalized glycogenosis or cardiac glycogen storage disease have been observed The first two were brother and a sister and have been previously reported

The tree of the W family is represented in FIGURE 2 The patient s father was born in southern Italy and the mother was born in the United States, b t her parents had emigrated from southern Italy and were first cousins Both parents had many siblings, some of whom had died in infancy of unknown causes

The first child Lucy was born in 1922 and delivery was normal A few weeks after birth the parents noted what they described as "heavy breathing" and, some time later the appearance of intermittent cyanosis, more marked during feeding The patient was admitted at the age of four mo ths in a moribund condition The infant died nine hours after admission

At autopsy the heart was greatly enlarged On microscopic examination the cardiac muscle had what w later called a typical lacework appearance, with much of the myocardial fiber replaced by glycogen. In the sections still available, evidence of glycogen deposition was found in other tissues A

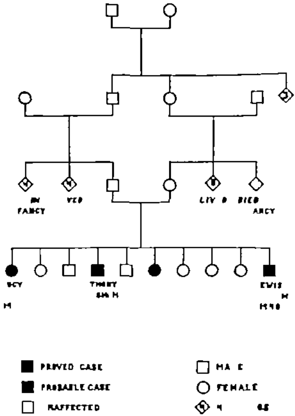


FIGURE 2 Family tree of the W family Reproduced by permission of *Pediatrics* [18]

diagnosis of diffuse rhabdomyomatosis of the heart was made at the time When, about ten years later cardiac glycogen storage disease was recognized as an entity the diagnosis was corrected.

Of the next eight siblings, two Anthony and Theresa, died at other hospitals at the age of three and one half and four months, respectively of an illness that in all ways resembled that of the first child The clinical and, in one case the autopsy records were unfortunately no longer available

Lewis was born to the same mother in 1948 as a result of the tenth pregnancy Pregnancy delivery and early neonatal period were uneventful At the age of 7 weeks the parents noted a peculiar type of "heavy breathing" that attracted their attention because it was similar to that of the 3 children who had died At the age of 2 months the infant began having spells of cyanosis, especially noticeable after feeding or when crying At the age of 3 months the patient was admitted for investigation. The heart was found enlarged in all diameters, the sounds were of poor quality and there was a soft blowing systolic murmur heard all over the precordium The liver was felt 3 cm below the costal margin the tip of the spleen was palpable. Because of the family history a



FIGURE 3 Roentgenogram of the chest in Lewis W (tube distance, 2 m) Reproduced by permission of *Pediatrics*.[14]

tentative diagnosis of cardiomegalia glycogenica was made. Cyanosis was present only on feeding in the first few weeks. The apical heart rate remained between 130 and 140 per min The patient deteriorated gradually his respirations became more rapid and labored cyanosis became constant He died after 33 days in the hospital

A roentgenogram of the chest 3 days after admission showed a globular heart enormously enlarged in all diameters (FIGURE 3) Electrocardiograms revealed a marked left axis deviation, which is very abnormal for a patient of this age markedly inverted T waves in leads II and III and diphasic in lead I The S-T segment was depressed in leads I and II Many ante mortem chemical investigations were carried out and were all negative. In particular glucose, galactose, and levulose tolerance tests were normal. The glycemic and lactic acid responses to injection of epinephrine were normal.

At necropsy the heart was enormously enlarged it weighed 148 gm as compared with the normal 29 gm It was globular and the enlargement was *symmetrical and primarily ventricular with disproportionately small auricles*, quite typical in appearance for this disease The ventricular walls were thickened and the myocardium paler and firmer than usual Microscopically the myocardium had the typical lacework appearance (FIGURE 4) each fiber hollow cylinder surrounded by thin, delicate cytoplasm, the nucleus lying free in the center due to the massive infiltration of glycogen The change in structure was so great as to make recognition of myocardial tissue difficult Striking changes were also found in skeletal muscle. The average diameter

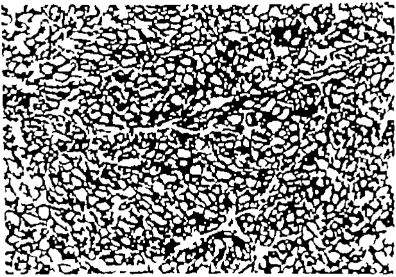


FIGURE 4 Lewis W heart muscle, showing central clear area in fibers and peripheral sarcoplasm Nuclei are found in center of fiber All fibers are involved (so-called lacework appearance) Hematoxylin and eosin ×100 Reproduced by permission of *Pediatrics* [19]

of the fibers was increased from 2 to 5 times moderately affected ones had rows of vacuoles lying between fibrillae, while in the larger fibers the entire cytoplasm appeared replaced by glycogen The amount of this polysaccharide varied greatly in different muscles. In virtually all other tissues, including smooth muscle, liver and others, glycogen was present in large amounts.

Results of post-mortem chemical studies showed that the glycogen content was highest in the heart (9 8 per cent) but not much lower in the tongue (6 6 per cent) psoas muscle (6.3 per cent) and liver (7 1 per cent) Normal values for glycogen content as given by Van Creveld vary from 0 07 per cent for the myocardium to 0 103 per cent for the hepatic tissues After other samples of the same tissues had been kept for 24 hours in the incubator at 37 5 C chemical analysis revealed that autoglycogenolysis had taken place in all instances, although the degree varied in the different organs.

The third patient we observed was D B the third child of the B family A previous sibling had suffered from the cardiac type of glycogen storage disease and was reported in the literature [1] She had died at the age of five and one half months The second child in the family was normal as was the fourth sibling The parents were both of English extraction, born in the United States, and not related

D B was born at our hospital and followed from birth which afforded an unusual opportunity to study the development of this disease On physical examination shortly after delivery the child appeared quite normal, had a good muscular tone, but was found to have a harsh murmur Roentgenogram of the chest on the fourth day of life showed the heart shadow to be greatly enlarged in all diameters However the electrocardiogram, done on the sixth day was considered normal

TABLE 2
POST MORTEM CHEMICAL STUDIES

D B aged 4 mo	Tissue content of glycogen	Structure analyses		Glucose-6-phosphatase activity
		1 6-Bonds in glycogen	Degradation of glycogen by phosphorylase	
	(percentages)	(percentages)	(percentages)	
Heart	7 1	6 4	33 3	—
Liver	6 0	7 2	37 5	Normal
Adductor	6 6	7 6	40 1	—
Tongue	8 2	—	—	—
Rectus	5 0	—	—	—
Diaphragm	2 5	—	—	—
Kidney	1 2	—	—	Normal
Brain	0 4	—	—	—

Performed by Gerty Cori.

The baby appeared to act and grow normally and he had no difficulty feeding *until the age of 6 weeks, when he began to have marked respiratory distress.* He had auscultatory rales all over the pulmonic areas and the liver was markedly enlarged He was considered to be in failure. After admission he improved considerably Digitalis was given The heart was markedly enlarged on X ray and the electrocardiogram showed inversion of the T waves and depression of the S-T segment in several leads. The changes continued progressively Many chemical tests were performed and were all within normal limits. In particular the glycemic response to epinephrine injection was quite normal Subsequent episodes occurred characterized by cyanosis, respiratory distress, and enlargement of the liver Feeding became difficult the patient became progressively worse and he died at the age of 3 months.

At necropsy the findings were similar to those of the other patient There was an enormously enlarged, globular heart Microscopically the appearance of the myocardium was quite typical of this disease and there was microscopic evidence of deposition of glycogen in all tissues of the body The appearance of the tissues and in particular of the striate muscle was in all ways similar t that of other patients

All tissues were frozen 90 min after death and were sent on several occasions by air in dry ice t Gerty Cori in St. Louis, Mo who performed all of the chemical analyses. The results are listed in TABLE 2.

All tissues investigated contained a large amount of glycogen The content of this polysaccharide was a little higher in the myocardium than in most tissues with the exception of the tongue but the figures are closely comparable in the liver tissue. There were 650 mg of free glucose per cent in the hepatic tissue on analysis, and another 150 mg formed after incubation of the liver tissue for 3 hours at 30° C. In the adductor striate muscle tissue there were 273 mg. of lactic acid per 100 gm. These results indicat that in both liver and muscle glycogen could break down, quite in keeping with previous studies. Glucose-6-phosphatase activity was determined in both liver and kidney tissue and was found t be normal Structure analyses of glycogen was

with the enzymatic method using alternately phosphorylase and the debranching enzyme amylo-1 6-glucosidase. The glycogen structure was considered normal.

Specimens from several similar cases have been subsequently analyzed by Gerty Cori and have yielded similar findings

Conclusions

It is evident, therefore, that so-called glycogen storage disease of the heart (Type II) is a separate and distinct entity among the diseases of glycogen storage. It is in reality a generalized disorder due to a metabolic error affecting carbohydrate metabolism throughout the body and not only in the heart. Cardiac enlargement is its major clinical expression and the cause of death. The so-called cardiac type of glycogen storage is incompatible with life for more than a few months, during which cardiac function continuously regresses The most plausible explanation is that of a mechanical death produced by an accumulation of glycogen in the myocardial fibers to a point where their contraction is impeded and effective cardiac action no longer possible.

It is interesting in this respect to note that in the glycogen storage disease called limit dextrinosis or Cori's disease, our Type III, characterized by an abnormal glycogen structure analysis by Cori in two cases[11] shows an excess of glycogen in the myocardium, as well as in the striate muscle The levels in the examined specimens have been less than 5 per cent of wet weight and, insofar as is known, patients have neither been in serious trouble clinically because of their cardiac function, nor have their electrocardiograms or roentgenograms been abnormal Apparently the percentage of glycogen in the myocardium must be above this level to cause symptoms when it reaches 7 per cent or above it becomes incompatible with cardiac function.

What metabolic defect leads to this generalized and abnormal glycogen accumulation in the myocardium and throughout the body in the cardiac type of storage disease has not been ascertained but the studies we have made thus far shed some light, in a negative way on this obscure subject On clinical grounds we knew the glycogenolytic function of the liver was preserved and now we have the measurement of glucose-6-phosphatase showing it is normal. The structure of glycogen is also normal in this type of disease There is reason to believe that glycogen can be utilized by the body in these cases because of a preserved capacity in the liver and the muscle to break down this polysaccharide and because of the absence of demonstrable accumulation of intermediate products of carbohydrate metabolism, such as pyruvate or lactate, or of any evidence pointing toward the combustion of large amounts of fat

The cardiac type of glycogen storage disease is hereditary and presumably it is transmitted as a recessive It is rare, as shown by the fact we have seen only 3 cases of this condition in two families at Babies Hospital in 37 years, and in more than 3000 autopsies, and only 20 cases have been recorded in the literature, although many more have been seen, but not reported Criteria can be listed as follows for establishment of this diagnosis (1) marked enlargement of the heart (2) death within the first year of life due to heart

failure (3) typical lacework appearance of histological sections of the myocardium resulting from massive deposition of stored material in all cardiac fibers and (4) post mortem chemical studies demonstrating a normal glycogen structure and preserved glycogenolysis in the liver and in the striate muscle

In the differential diagnosis other conditions giving rise to cardiac hypertrophy in infancy should be considered such as endocardial fibroelastosis, viral myocarditis, and aberrant origin of the coronary artery Electrocardiographic findings suggestive of myocardial damage can be seen in many of these conditions and the roentgenographic appearance is not conclusive. A biopsy of the striate muscle showing the histological and histochemical evidence of excessive glycogen deposition with the characteristic degeneration of muscle fibers that accompanies it is highly suggestive of this disease.

It should also be kept in mind that there are many other circumstances in which excess glycogen is present in the myocardium, but in a spotty and not generalized distribution Rhabdomyoma of the heart, so-called cardiomegalia glycogenica circumscripta, and cases of sudden death in which excessive but localized accumulation of glycogen is found in the myocardium after death are among these conditions There is evidence that, when myocardial cells are destroyed by a variety of agents, infiltration with glycogen occurs This is obviously due to a different mechanism and should not be confused with the unknown metabolic defect that leads to excessive glycogen storage in all tissues of the body including the myocardium

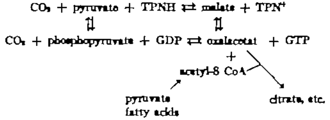
In conclusion the diseases of glycogen storage, in particular those of the cardiac type, constitute a very obscure field in which a faint glimmer of light has been spread by the collaboration of the clinician, the pathologist, the cardiologist and primarily the chemist Much more work remains to be done and, if further advances are to be made in this confused field they will be due to continued cooperation among disciplines In particular application by the chemist of his specialized knowledge and techniques will undoubtedly prove highly rewarding

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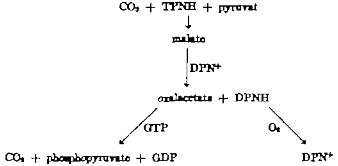
SCHEME 1

POSSIBLE EFFECTS O CO_2-FIXA REACTIONS ON OXIDATIVE MECHANISMS



SCHEME 2

POSSIBLE ROLE OF CO_2 FIXATION IN THE FORMATION O PHOSPHOPYRUVATE FROM PYRUVATE



It is possible to visualize a role for the CO_2-fixation reactions in the formation of phosphopyruvate from pyruvate, as shown in SCHEME 2. In this proposed sequence pyruvate is converted to phosphopyruvate by the combined action of the two CO_2-fixing enzymes, although a number of other enzymes are also required to supply TPNH and GTP, to remove DPNH, and to oxidize malate to oxalacetate.

The net reaction is essentially the conversion of pyruvate and GTP to phosphopyruvate at the expense of the oxidation of TPNH

$$\text{pyruvate} + \text{GTP} + \text{TPNH} + \tfrac{1}{2}O_2 \rightarrow \text{phosphopyruvate} + \text{GDP} + TPN^+ + H_2O \quad (3)$$

This reaction sequence provides a mechanism for the reversal of the pyruvic kinase reaction

$$\text{phosphopyruvate} + \text{ATP} \rightleftarrows \text{pyruvate} + \text{ATP} \quad (4)$$

Reaction 4 has been shown to be reversible, but SCHEME 2 was proposed as a more feasible pathway for phosphopyruvate formation on thermodynamic grounds.[4, 5] The equilibrium constant of REACTION 4 lies far to the right while the equilibrium of Reaction 3 should favor phosphopyruvate synthesis. Although GTP is believed to be the phosphate donor in SCHEME 2, it can be

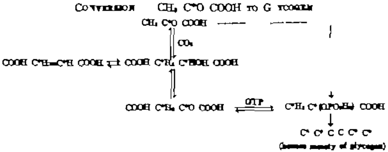
formed from ATP and GDP by the nucleoside diphosphokinase reaction

$$ATP + GDP \rightleftarrows ADP + GTP \quad (5)$$

The present discussion will attempt to examine the available data with occasional references to SCHEME 2 to see if the concept embodied in SCHEME 2 has any validity It will be of particular interest to consider various tissues independently

Data obtained by the use of isotopically labeled substrates support the belief that CO_2 fixation is involved in the formation of liver glycogen from substrates at the level of lactate or pyruvate It has been well established that isotopically labeled CO_2 is incorporated into the 3 and 4 positions of the hexose moiety of liver glycogen In addition, Topper and Hastings[1] and Shreeve[11] showed that, in the conversion of 2-pyruvate-C^{14} t glycogen by liver slices, the distribution of C^{14} in glycogen is that expected if the pathway involves a symmetrical intermediat such as fumarate, as shown in SCHEME 3

SCHEME 3



If pyruvate labeled as indicated is converted to malat by CO_2 fixation, the label will be randomized between the two noncarboxylic carbon atoms by fumarase, with the result that th phosphopyruvate formed subsequently will bear a 2 and 3 labeling, and glycogen hexose a 1 2, 5 and 6 labeling If pyruvate is converted directly to phosphopyruvate without passing through symmetrical intermediate, the phosphopyruvate will be labeled only in the 2 position and glycogen hexose only in the 2 and 5 positions The actual experimental results[1, 11] showed that the glycogen was labeled almost equally in the 1 2, 5 and 6 positions supporting the first of these two pathways for phosphopyruvate synthesis Unfortunately as often is the case in isotopic experiments, the results can also be interpreted as the result of exchange reactions that do not necessarily reflect net synthesis. In SCHEME 3 if phosphopyruvate is formed directly from pyruvate (dotted line) equilibration of the phosphopyruvat with the dicarboxylic acids can lead t a randomization of the label in the phosphopyruvate and a subsequent formation of glycogen with the observed labeling

W have investigated the possible relationship of the CO_2-fixing reactions to phosphopyruvat formation in a somewhat different fashion. Pennington

TABLE 1
FORMATION OF PHOSPHOPYRUVATE FROM PYRUVATE

Incubation time	Phosphopyruvate	Phosphoglycerate
min	µmoles	µmoles
0	0	0
30	0 25	0 13
60	0 47	0 25
90	0 74	0 66

Incubation mixture contains glucose 6-P dehydrogenase, malic enzyme, diaphorase, PEP carboxykinase, and malic dehydrogenase, as well as glucose 6 P TPN DPN, ITP Na pyruvate MnCl₂ and NaHCO₃ Total volume = 2 4 ml temperature, 37° C

TABLE 2
PHOSPHOPYRUVATE FORMATION BY DICARBOXYLIC ACID PATHWAY

System	Phosphopyruvate	Phosphoglycerate
	µmoles	µmoles
Complete	0 41	0 16
TPN	0 08	0
DPN	0	0
ITP	0	0
Pyruvat	0	0

Conditions and components were the same as those in TABLE 1 Time, 60 min

and I first tried to accomplish the formation of phosphopyruvate from pyruvate by an *in vitro* system composed of the various enzymes and substrates that had been suggested as taking part in such a sequence of reactions. These attempts have been successful, but will be touched on only very briefly As shown in TABLE 1 when malic enzyme and PEP carboxykinase are incubated with pyruvate, CO_2 a phosphate donor and the required subsidiary enzymes and cofactors, phosphopyruvate and phosphoglycerate, are formed TABLE 2 shows that the various cofactors and substrates are all necessary for the reaction to proceed These experiments demonstrate that it is possible to

TABLE 3
ENZYMATIC MEASUREMENTS ON RAT TISSUES

Tissue	PEP carboxykinase*	Malic enzyme*	Pyruvic kinase*
Skeletal muscle	0 02	0 5	52
Brain	0 14	1 6	9 3
Heart	0 21	1 7	7 7
Kidney	2 9	1 5	2 8
Liver	1 4	2 3	1 3

Activity = µmoles/min /10 mg protein.
Assays were carried out on extracts of acetone powder of tissues. Pyruvic kinase and malic enzyme were assayed spectrophotometrically at 30° C. PEP carboxykinase at 38° C by $C^{14}O_2$ exchange with oxalacetate

link together purified enzymes to carry out the dicarboxylic pathway of phosphopyruvate formation. Stated in other terms, this pathw y has been shown to be feasible in a kinetic sense.

The foregoing experiments were performed with enzymes obtained from a variety of sources. If the dicarboxylic acid path y has any abidity all these enzymes must be present in reasonabl amounts in a single tissue We were interested particularly in the two CO_2-fixing enzymes, since other evidence was available to indicate that the remaining enzymes have a wide distribution and were probably present in all the tissues tested Accordingly Hsu and I have measured the amounts of malic enzyme and PEP carboxykinase in arious tissues. TABLE 3 shows the relati e amounts of these enzymes in extracts of acetone powders of five rat tissues. Pyruvic kinase has also been included, since it constitutes a possible alternate route for phosphopyruvate synthesis The most significant findings seem to be these

(1) PEP carboxykinase is found in liver and kidney The small amounts found in the other tissues approach the sensitiv ty of the assay method, although the values for heart and brain may be significant

(2) Malic enzyme has a fairly uniform distribution in the tissues tested, with the exception of a lower value for skeletal muscle Even in muscle th value is significant although in earlier studies using a different assay procedure, malic enzyme had not been detected in significant amounts

(3) Pyruvic kinase is found in all tissues examined, but there is a large variation with skeletal muscle containing about forty times as much of this enzyme as is contained in liver

The quantitative estimation of enzymes in tissues has many pitfalls, and the values obtained may vary with such factors as the handling and extraction of the tissue, the assay procedure, the presence of interfering enzymes, and the species of animal employed A variety of procedures has been used in the determination of these three enzymes in rat, rabbit, and chicken tissues and although the values obtained vary somewhat depending on the above factors, in all cases the general pattern is that shown in TABLE 3 I believe that these relationships will prove to be essentially correct with the following species differences the CO_2-fixing enzymes are considerably higher on a specific activity basis in avian liver disparity in pyruvic kinase content of various tissues is even more marked in rabbit and chicken tissues than is shown for rat tissues in TABLE 3

How do these distribution studies agree with the previously outlined roles of the CO_2-fixation reactions and with other knowledge of the biochemistry f the various tissues The ability of the liver to form glycogen from lactate pyruvate, and certain amino acids is well known, and there is also considerabl evidence that the kidney can produce glycogen or glucose from similar substrates [22]- If glycogen is formed via the dicarboxylic acid pathway these tissues should be well supplied w th malic enzyme and PEP carboxykinase. Indeed, we find that these are the best and perhaps only sources of PEP carboxykinase and that malic enzym is also present in considerabl amounts in these tissues. Conversely the pyruvic kinase in these tissues is the lowest of all those examined

It might be pointed out that phosphopyruvate synthesis by a pathway such as the dicarboxylic acid route would be largely negated by the presence of an excess of pyruvic kinase, as this enzyme would reconvert phosphopyruvate to pyruvate in the presence of ADP and a mechanism for the regeneration of ADP from ATP. There is little evidence that skeletal muscle or brain can form glycogen at any appreciable rate from substances such as pyruvate or lactate. These tissues contain little or no PEP carboxykinase, but are the highest in pyruvic kinase content. CO_2 fixation may still play a role in these tissues in the synthesis of the dicarboxylic acids needed for the operation of the Krebs cycle, since malic enzyme is present in both cases.

In a qualitative way, then, the results of the enzyme distribution studies in the four tissues just mentioned support the hypothesis that CO_2 fixation plays a part in glycogen synthesis from 3-carbon acids. The situation with regard to the heart is less satisfactory. On the basis of the enzymatic studies one would conclude that the conversion of 3-carbon acids to glycogen by the dicarboxylic acid pathway must be slow or perhaps absent. Other types of evidence suggest that lactate may be converted to glycogen in heart, although this point cannot be regarded as settled. The heart has a substantial glycogen content and utilizes lactate readily as a substrate. Observations that lactate could serve as a major substrate for isolated heart preparations, meanwhile maintaining th glycogen content, led to suggestions that lactate might be converted to glycogen.[17]

As was made clear in the original reports, the results can be interpreted equally well as a sparing action of lactate on glycogen and cannot be considered as conclusive evidence for glycogen synthesis from lactate. Stadie *et al.*[18] showed that slices from rat heart could form glycogen from glucose, but they were unable to demonstrate glycogen formation with lactate pyruvate or alanine as substrates. Perhaps the most substantial evidence that heart may form glycogen from 3-carbon precursors is provided by the experiments of Lorber *et al.*[19] on the incorporation of $C^{14}O_2$ into the glycogen of the isolated cat heart when incubated with glucose and lactate. These workers found that heart glycogen acquired a substantial labeling under these circumstances in a relatively short time. Their results suggest that the isolated heart has the capacity to synthesize glycogen by the dicarboxylic acid pathway, by reversal of the pyruvic kinase reaction, or by both mechanisms. It should be noted, however, that it is possible to suggest mechanisms by which $C^{*}O_2$ could enter glycogen by exchange reactions that do not lead to net synthesis of glycogen.

It is unlikely that a species difference is involved in the apparent contradiction between the enzymatic and perfusion experiments since cat, dog, chicken, and rat heart preparations all give essentially the same results in enzymatic studies. At present the question of glycogen formation from 3-carbon acids in heart must be regarded as unsettled. However, malic enzyme is present in all heart tissues tested in relatively high amounts; therefore, CO_2 fixation may play a role in this tissue in the formation of the 4-carbon dicarboxylic acids needed for oxidative reactions.

The intracellular distribution of the same three enzymes is of some interest.

TABLE 4

INTRACELLULAR DISTRIBUTION OF ENZYMES CONCERNED WITH PHOSPHOPYRUVATE FORMATION

Cellular fraction	Malic enzyme	PEP carboxykinase	Pyruv. kinase
Mitochondria	−	+	±
Microsomes	−		+
Supernatant	+	±	+

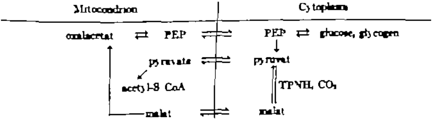
Fractionations of chicken liver homogenates carried out in 0.25 M sucrose.

Fractionation studies of homogenates of chicken liver by the usual centrifugal techniques are summarized in TABLE 4. As indicated, malic enzyme is almost completely localized in the supernatant fraction, while PEP carboxykinase is found mainly in the mitochondrial fraction. A small but variable amount of PEP carboxykinase appears in the supernatant, probably because of mitochondrial destruction during the handling procedures. Pyruvic kinase appears to be distributed between the microsomal and supernatant fraction; a small amount is also found associated with the mitochondria, probably as contaminant.

According to these findings, the general relationship between the enzymes of the mitochondrion and the cytoplasm might be represented as shown in SCHEME 4. According to this hypothesis the conversion of pyruvate to phos-

SCHEME 4

RELATIONSHIPS OF MITOCHONDRION AND CYTOPLASM



phopyruvate would involve both the cytoplasmic and mitochondrial elements of liver cells, with the pyruvate-to-malate conversion occurring in the cytoplasm and the further conversion of malate to phosphopyruvate occurring within or on the mitochondrion.

Furthermore, mitochondria should be able to form phosphopyruvate from substances that yield oxalacetate. Many years ago Kalckar[36] showed that kidney particulates could form phosphopyruvate from malate. Bartley[37a] and Bandurski and Lipmann[37] have shown that liver mitochondria also form phosphopyruvate from dicarboxylic acids, and we also have found this to be true.

In view of the uncertainties concerning the possible formation of phosphopyruvate in heart, it would be of interest to see if heart mitochondria could

also form phosphopyruvate from malate. Thus far our efforts in this direction have been completely unsuccessful.

Up to this point the consideration of CO_2-fixing reactions in phosphopyruvate and glycogen synthesis has been limited to a discussion of qualitative studies. We now have attempted to secure more quantitative information in the case of one tissue, chicken liver. Even if hormonal, dietary, and other factors are neglected, the following information is required in order to determine whether the dicarboxylic acid pathway is sufficiently active to account for phosphopyruvate and glycogen synthesis: (1) the rate of glycogen synthesis, (2) the concentration of the enzymes involved, (3) the relationship between substrate concentration and reaction velocities of these enzymes, and (4) the *in vivo* concentration of the substrate or substrates. As will be readily seen, it is difficult to find a metabolic pathway for which this type of detailed quantitative information is available; we do not have all of these data for the dicarboxylic acid pathway. However, it may be of interest to present briefly the available information, as it permits certain tentative conclusions concerning the route of phosphopyruvate synthesis, and also because it is illustrative of a type of problem that will appear more frequently in biochemical studies as the quantitative evaluation of potential pathways becomes necessary.

The first question concerns the rate of glycogen synthesis. Even this question cannot be answered with certainty. In typical experiments[33] in which fasted rats are given sodium lactate, the amount of glycogen laid down in a two-hour period is equivalent to perhaps 1 μmole of triose per minute per gram of liver. The rate in liver slices is slower, perhaps about 0.25 μmole of triose per minute per gram.[34] These values can be considered only as rough approximations since they are calculated from a single time interval and since the glycogen may not all arise from lactate.

The concentrations of pyruvic kinase, malic enzyme, and PEP carboxykinase in chicken liver are shown in TABLE 5 with the results expressed as μmoles of activity per minute per gram of liver at 37° C. The second column from the right shows the mean of the determinations on a series of 10 chickens. For practical reasons each of the three enzymes was measured in the opposite direction from phosphopyruvate synthesis, and it was necessary to calculate the rate in the direction of phosphopyruvate synthesis. This was accomplished by the use of ratios of the velocities of the forward and back reactions obtained

TABLE 5

ACTIVITIES OF ENZYMES CONCERNED WITH PHOSPHOPYRUVATE SYNTHESIS (CHICKEN LIVER)

Enzyme	Assay	μmoles/min./gm.*	Maximum rate in direction of phosphopyruvate synthesis (calculated)†
Pyruvic kinase	Pyruvate formation	7.2	0.05
Malic enzyme	TPNH formation	5.0	1.55
PEP carboxykinase	$C^{14}O_2$ into oxalacetate	10.9	25.5

* Activities corrected to 37° C.
† Calculated from previous column using ratios of forward and backward velocities.

from experiments with purified enzymes T illustrate pyruvic kinase was determined by a spectrophotometric assay as shown in REACTIONS 6 and 7

$$\text{phosphopyruvate} + \text{ADP} \xrightleftharpoons{\text{pyruvic kinase}} \text{pyruvate} + \text{ATP} \quad (6)$$

$$\text{pyruvate} + \text{DPNH} \xrightleftharpoons{\text{lactic dehydrogenase}} \text{lactat} + \text{DPN}^+ \quad (7)$$

For a number of reasons it is not possible to measure REACTION 6 in the reverse direction in crude extracts Therefore the ratio of the initial forward and back velocities of this reaction was measured using highly purified pyruvic kinase John McQuate carried out similar experiments in our laboratory and found that the ratio of the forward and back velocities is about 140 at pH 7 4 despite the fact that the K_{eq} of the reaction at the same pH is approximately 6000 Similar experiments ere carried out with malic enzyme and PEP carboxykinase to obtain the ratio of the initial velocities in the two directions. These ratios were then utilized to calculate the values shown in the right hand column of TABLE 5 These alues represent the maximum attainable velocities of the three enzymes in the direction of phosphopyruvate synthesis under optimal conditions

The values shown in TABLE 5 are calculated on the basis of maximum initial velocities under optimal conditions of substrate concentration. *In vivo* the conditions may be less favorable and the actual velocities will probably be less than those shown.

The values shown in TABLE 5 are subject to considerable error but probably establish the general order of magnitude. Even allowing for the probable errors, it would appear that the pyruvic kinase in liver cannot account for an appreciable part of the phosphopyruvate synthesis in that tissue. There is possibly enough malic enzyme to account for phosphopyruvate synthesis, although the maximum rate attainable, 1.55 μmoles, is fairly close to the estimated rate required (1 0 μmole) The concentration of PEP carboxykinase appears to be in considerable excess

The enzymatic velocities discussed above are maximum initial velocities the actual rates *in vivo* will depend upon the concentration of substrate available and on other factors. The relationship of reaction velocity and substrate concentration is reflected in the Michaelis-Menten constant K_s. These values for the enzymes under discussion are given in TABLE 6, insofar as they are available. One of the most striking points is the very high K_s for pyruvate of pyruvic kinase ($9 \times 10^{-}$ M) This value may be contrasted with the K_s of pyruvate for malic enzyme which is very much lower (3×10^{-4} M) and probably much more in accord with physiological concentrations of pyruvate. The K_s for CO_2 for malic enzyme is not known with any precision, but experiments given by Ochoa[28] and some of our own data indicate that the K_s is quite high. If this is true it poses a formidable obstacle t the dicarboxylic acid pathway unless there exists some unrecognized mechanism for activating or concentrating CO_2 at the site of enzyme action. It is interesting to note that the same sort of dilemma exists in the photosynthetic fixation of C with ribulose diphosphate for which the K_s for CO_2 with the purified e

TABLE 6

K_m VALUES TO SUBSTRATES INVOLVED IN PHOSPHOPYRUVATE SYNTHESIS

Enzyme	Substrate	K_m
Pyruvic kinase	Pyruvate	9×10^{-4} M
	ATP	8×10^{-4} M
Malic enzyme†	Pyruvate	3×10^{-4} M
	TPNH	Very low
	CO_2	6×10^{-4}(?)
PEP carboxykinase†	GTP	Very low
	Oxalacetate	Low

Purified from rabbit muscle
† Purified from avian liver

is very high When the naturally occurring concentrations of enzyme are considered this reaction can hardly account for the rate of photosynthetic CO_2 fixation [20]

The tentative conclusions that may be drawn concerning the role of the CO_2-fixing reactions carried out by malic enzyme and PEP carboxykinase in animal tissues seem to me to be as follows malic enzyme has a very wide distribution in animal tissues and potentially can participate in the oxidative reaction of these tissues by supplying dicarboxylic acids. In addition, in liver and kidney there is evidence to suggest that malic enzyme and PEP carboxykinase form part of a mechanism for the synthesis of phosphopyruvate from pyruvate during glycogenesis Quantitative studies of the amounts of enzymes present and their kinetic properties are not sufficiently detailed to permit a definitive answer to the problem of whether phosphopyruvate formation by a pathway involving CO_2 fixation is the major route of synthesis

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ENERGY-GENERATING SYSTEMS

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It is generally accepted that cardiac hypertrophy and failure occur as the result of factors connected with an increased load to the muscle and that the hypertrophic failing muscle is inefficient This, however is illogical since, by increasing its size the organ paradoxically increases its difficulties for more efficient oxygenation. The heart muscle, although in general an aerobic organ, is fairly well endowed with glycolytic enzymes. I wonder if the increase in size when the oxyhemoglobin supply is lowered is the primary defect and if it results in adaptation to a more anaerobic condition at the cell level with a corresponding increase in glycolytic enzymes. I wonder also about the possibility that the control mechanism for glycogen storage in the heart, under normal and abnormal conditions, leads to cardiac failure

This latter problem is of particular interest in view of the recent discovery and identification of an adenylphosphate anhydride as the co-factor for activation of liver phosphorylase[1] and of the new pathway for glycogen synthesis[2] from uridine diphosphate glucose (UDPG) Is it possible that glycogen is stored by the UDPG enzyme system, that glycolysis proceeds physiologically under the control action of glucagon, and that the phosphorylase becomes maximally activated by adrenaline as an emergency mechanism? Is glycogen in the muscle less dynamic than glycogen in the liver? What about heart glycogen does glycogen influence tissue metabolism and thus contributes more than just a storage function? Some of these question have been asked previously and, biochemically speaking, there does seem to be a marked difference between muscle and liver phosphorylase however a definite answer is yet to be given

For many years there have been reports in the literature on nonorthodox nucleotides, the tri- di- and monophosphates of adenosine (ATP ADP and AMP) for example the ATP type II of Banga or the adenine dinucleotide of Kiessling and Meyerhoff[3] that appears to resemble if not to be identical with the co-factor for fermentation of Ohlmeyer[4] Are these cofactors, or similar ones, perhaps the heart nucleotide H, important to cardiac muscle or to its regulation, whether or not at the carbohydrate level? In regard to this, an initial ATP splitting in muscle contraction has been challenged recently However the direct transfer of phosphate from 1 3-diphosphoglycerate to creatine to form phosphocreatine without the intermediate action of an adenine nucleotide has been shown recently Perhaps, since the enzyme[5] that produces the reaction 1 3-diphosphoglycerate $\rightarrow$ creatine phosphate seems to be lacking in heart muscle, this organ should be used to test whether ATP is split initially during contraction Heart muscle has much less phosphocreatine and ATP than has skeletal muscle[1] and would therefore, provide a better experimental model It should be remembered that there was splitting of an unidentified organic phosphate[6] during a single muscle twitch, although

the ATP and creatine phosphate concentration did not change Therefore the 1 3-diphosphoglycerate → creatine phosphate reaction, or a similar one may interfere seriously with th ATP and creatine phosphate balance studies

Since some derangements of cardiac function appear to be initially related to metabolic, rather than to mechanical defects as in beri-beri and in hypothyroidism, is it possible that there are more initial metabolic defects in the cardiac muscle, both before and at failure than we are aware of at present? In my opinion we should not expect to find early dramatic changes, at least in biochemical parameters, in conditions which develop gradually I am sure *that a continuous, for example a 5 per cent, change in metabolic regulation* would have a very serious effect in the long run however it is exceptional to be able to measure biochemical reactions in crude samples within a 5-per cent range.

Thiamine therapy has been recommended on the basis of better response of the partially thiamine deficient heart to cardiac glycosides in the presence of additional thiamine.[11] Certainly there are some clinical cardiopathies labeled idiopathic which occur mostly in young or middle-aged people these patients, although they are without apparent mechanical defect, eventually display cardiac dilatation and failure Perhaps a further indication of individual susceptibility is the apparent fundamental defect in alcohol cardiopathy and, for that matter the extreme variability of the liver in resisting cirrhosis whether due to alcohol or other causes

The hormone thyroxine appears to influence both glycolysis and respiration, and the interdependence of these processes is *well known* (Pasteur and Crabtree effects)

Therefore, the uncoupling effects of thyroxine upon oxidative phosphorylation, the interesting recent observation[13] that the swelling of mitochondria produced by this hormone is dependent on the redox state of the mitochondria (since under anaerobic conditions or in the presence of cyanide there is no effect) the possibility of using this observation as an experimental testing model for permeability effects of digitalis, and the many observations on inhibitory effects of thyroxine on dehydrogenases, transhydrogenases, creatine kinase, etc *will not* be discussed here.

Recently it has been observed that this hormone thyroxine inhibits completely acyl phosphatase,[14] an enzyme that, among other substrates, can dephosphorylate 1 3-d phosphoglycerate[1] (1 3-DPGA) Recent work in our laboratory with highly purified heart and brain carbamyl phosphatase[1 17] appears to indicate that carbamyl *phosphatase and acyl phosphatase are the same* enzyme.[18] This enzyme which is probably the smallest thus far recorded (M W = 10,000 or less) may be important in both carbohydrat and nitrogen metabolism

I should like to stress the possible importance of this observation since there are indications for a self-regulatory cycle at the phosphoglycerate level as first suggested by Rapoport.[19] Whether this hormone affects the phosphoglycerate cycle shown in FIGURE 1 remains to be investigated. Recen it was observed in our laboratory that crude muscle extracts can conv

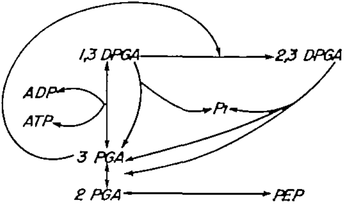


FIGURE 1 Phosphoglycerate cycle.

most quantitatively 3-phosphoglycerate to 2 3-diphosphoglycerate [24] I am not certain that this is a physiological effect—perhaps it is only an *in vitro* artifact however we are searching for alterations that may lead to the better understanding of heart failure

A block in the pyruvic kinase (or enolase) reaction, for example, or simply low levels of inorganic phosphate and ADP could result in accumulation of 3-phosphoglycerate (3-PGA) This, in turn, would stimulate the conversion of 1 3-DPGA to 2 3-diphosphoglycerate (2 3-DPGA) Although the 2 3-DPGA would stimulate the phosphoglycerate mutase reaction,[25] this would result in further accumulation of 3-PGA since the equilibrium is in favor of 3-PGA formation[26] and thereby further 2 3-DPGA accumulation Furthermore, 3-PGA inhibits the dephosphorylation of 2 3-DPGA to monophosphoglycerates [28] However once the block is removed, the accumulated 2 3-DPGA would increase the activity of the 3-PGA mutase and therefore, glycolysis. At the same time, inorganic phosphate and monophosphoglycerate would be formed via the 2 3-DPGA phosphatase [23]

Although we have demonstrated 2 3-DPGA accumulation in many tissues,[24] it is not known whether any or all tissues are under such regulatory mechanism.

Perhaps the high concentration of 2 3-DPGA in the human erythrocyte[29] and its variations in disease reflect such a mechanism (2 3-DPGA may or may not perform any other role in erythrocyte viability)

It is likely that the mixed *in vitro* effects thus far demonstrated with thyroxine will be shown to have a complex result in the living animal, as anticipated for a long time by physiologists

It must be remembered that most of our chemical methods are not adequate to reveal small but perhaps important differences between normal and abnormal tissues and that, to my knowledge, it is very difficult to visualize experimental heart failure comparable to that in the human patient This clearly indicates the need for a careful quantitative study of biochemical parameters in the human normal and abnormal myocardium. Post-mortem material may be useful in such studies since a number of components, particularly some enzymes, are relatively stable.

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CONSERVATION OF ENERGY IN CARDIAC MUSCLE*

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For the past few years our laboratory has been engaged in the systematic examination of the biochemical pathways in the normal and failing heart for evidence of a biochemical lesion that could be implicated in the pathogenesis of cardiac failure. Since the ability of the heart to perform work depends upon the intactness of all of the biochemical reactions that lead from the oxidation of substrate to the final contractile process, it seemed possible that interruption of this sequence at any point might lead to cardiac failure. This paper reviews the evidence that has been obtained regarding the energetics of the myocardium in the dog and in man under normal conditions and under two conditions that may lead to congestive heart failure namely thyrotoxicosis and severe valvular disease.

Metabolic processes in heart muscle may be divided into three general phases (1) energy liberation (2) energy conservation, and (3) energy utilization. These are presented diagrammatically in FIGURE 1. The phase of energy liberation is visualized as including those reactions by which the carbon—carbon and carbon—hydrogen bond energy of oxidizable substrate is liberated as free energy. In this phase cardiac fuel which consists chiefly of free fatty acids (NEFA) glucose lactate and pyruvate is extracted from the coronary blood and is metabolized to a common intermediate, acetylcoenzyme A. The acetyl group condenses with oxalacetate to yield citrate that is then degraded stepwise through the well-known reactions of the Krebs tricarboxylic acid cycle to yield 8 hydrogen atoms or electrons ($H = H^+ + e$) which represent the energy content of the acetyl fragment and are equivalent to the 4 hydrogen atoms associated with the acetyl group plus 4 that come from water added to cycle intermediates in effecting the oxidation of the fragment. The function of the glycolytic lipolytic, and citric acid cycle enzymes is thus to convert the bond energy of substrates available to the cardiac muscle cell into energy-rich electrons for transport to oxygen. This phase of energy liberation merges with the next phase of energy conservation through the mechanisms of oxidative phosphorylation, some of which occur at the substrate level however the most important (quantitatively) of them occur in association with the electron flow along the hydrogen transport chain. This is depicted in FIGURE 1 as coupling the phosphorylation of adenosine diphosphate (ADP) to the oxidation of the cytochrome enzymes. With an average P O ratio for carbohydrate or fatty acids of about 3 0, which is well validated, the efficiency of conversion of the chemical bond energy of substrate to adenosine triphosphate (ATP) appears to range from 60 to 66 per cent. Creatine phosphate

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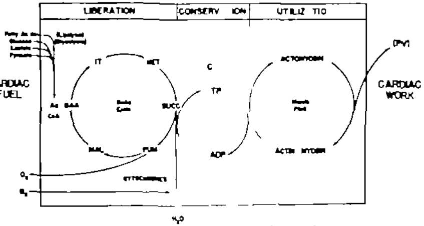


FIGURE 1 Schema of energetics in cardiac muscle

(CP) stores in cardiac muscle are relatively small (equivalent to 12 to 15 mg. per cent P) but provide some high energy phosphate buffer capacity by way of the enzyme transphosphorylase

The third phase of cardiac energetics is that of energy utilization. The high-energy phosphate bond energy of ATP is channeled int the contractile mechanism and results in the performance of mechanical work Although there is considerable controversy about the model that best represents the *contractile system*,[7] *the model* presented in FIGURE 1 has the advantage of being simple. In this system, actin (fibrous) plus free myosin are postulated to combine in diastole under the influence of ATP to yield actomyosin and ADP Excitation of the membrane at the time of electric systole then stimulates (possibly via K^+ shift) the energy-rich and fully extended actomyosi to dissociat into its component parts with shortening of the myofibril and the production of mechanical work. *The work efficiency of this phase* may be as high as 60 per cent, resulting in an over-all work efficiency in heart muscle of 35 to 40 per cent.

From the evidence t hand some years ago[4] we elaborated a working hypothesis that postulated that congestive heart failure of the high-output type seen in associatio with anemia, beriberi, and thyrotoxicosis, was due to defect in myocardial energy production (including the phases of energy liberation and/o conservation) whereas congestive heart failure of the low-output type secondary to valvular disease hypertension, or coronary artery disease was due to a defect in energy utilization, secondary to an inability of the contractile proteins to assimilate phosphate-bond energy or to associate and dissociate properly in the cardiac cycle I th light of this hypothesis w shall examine the data that have been obtained in the interim W shall emphasize the events that occur during the phases of energy liberation and conservatio in normal heart muscle and in heart muscle from animals and man with thyrotoxicosis and low-output congestive heart failure.

Metabolism of Normal Cardiac Muscle

Much evidence is available to indicate that cardiac muscle[6, 7] utilizes a variety of substrates as sources of energy including glucose lactate pyruvate fatty acids and to a lesser extent acetate ketone bodies, and amino acids The extent to which each substrate contributes to the energy requirement of the heart *in vivo* is influenced by its concentration in arterial blood, as well as by the state of nutrition and the endocrine balance of the organism In addition, since data obtained *in vitro* on the oxidative disposition of substrates by cardiac muscle have been applied to the intact heart, it should be pointed out that the behavior of cardiac muscle slices *in vitro* differs in several respects from that of the intact heart It has been shown previously[6] that the decay of the pyruvic oxidase system in cardiac muscle slices from rats and ducklings is a logarithmic function of the time of incubation at 37°C Only at very short time intervals after initiation of the incubation of previously chilled slices could Q_{O_2} values somewhat comparable to those obtained in intact mammalian hearts be obtained Additional comparative data on the extraction of pyruvate lactate and glucose by cardiac muscle *in vitro* and *in vivo* are presented in FIGURE 2 Cardiac muscle slices from well-fed ducklings were prepared as previously described and incubated in phosphate-saline medium with glucose lactate and pyruvate *separately* at various concentrations from 10 to 120 mg per cent for 1 hour in an atmosphere of oxygen The dependence of rate of extraction upon arterial level that is, initial concentration in the medium, is shown in the left hand plot For comparison with this, the extraction of these three substrates from arterial blood by intact dog

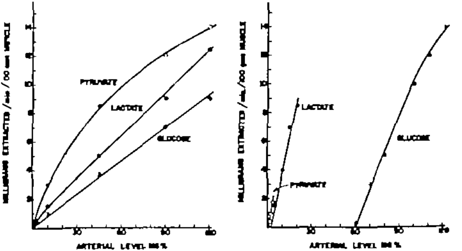


FIGURE 2 Extraction of glucose lactate and pyruvate by cardiac muscle *in vitro* and *in vivo*. The left hand diagram describes the behavior of cardiac muscle slices incubated with these respective substrates at various concentrations in phosphate saline medium with an oxygen atmosphere for one hour The right hand diagram describes the behavior of the intact dog heart at various concentrations of these substrates in arterial blood

myocardium as a function of arterial [illegible] tration is shown in th right-hand plot The latter data w re obtain [illegible] athet[illegible] tion of he lth postabsorptive and postprandial [illegible] tion rates er [illegible]lculated from the product of the arterio[illegible] ces for ea h substr te nd the coronary blood flow measured by th [illegible] technique

Certain differences in the extra[illegible] heart muscle *in vivo* and *in vitro* are immediatly [illegible] tion of substrate begins at zero concentration and [illegible] of initial oncentration of glucose and lactate this is not [illegible] tracted most rapidly at low concentrations [illegible] there is d finit threshold of utilization for each substrate Although [illegible] ery lose t zero for pyruvate (0.6 ± 0.2 mg per cent) nd la[illegible] 2.5 ± 0.1 mg per cent) the threshold is considerably higher for gluc[illegible] (5[illegible] ± 6 mg per cent) Above the threshold the extraction rates [illegible] functions of their respective arterial concentrations in the ranges commonl[illegible] ountered in the postabsorptive dog It is noteworthy that these lines w th the exception of that for pyruvate, have steeper slopes than those observ d [illegible] ardia muscle slices At levels of blood glucose about 100 mg per cent the xtraction curve for glucose becomes curvilinear approaching a maximum extraction of 25 mg /100 gm. muscle/min. at blood sugar values greater than 250 mg per cent.

The threshold phenomenon for extraction of glucose and the 3-carbon acids by intact heart is apparently lost in the slice That this threshold behavior of intact myocardium is a function of an intact cellular membra e is suggested by (1) the loss of this behavior in the slice, and by (2) modification of the threshold for glucose by changes in endocrine balance in the whole animal. Ungar *et al*[12] and Goodale *et al*[13] ha e show that the threshold for the extraction of glucose is markedly elevated in diabetes mellitus and restored toward normal by insulin treatment In normal dogs treated with large doses of insulin, Hackel has shown that the threshold for the extraction of glucose can be lowered to 10 ± 3 mg. per cent

Another important point should be made regarding the metabolic beha ior of intact heart muscle as opposed t cardiac muscle slices In the former the extraction and oxidation of substrate are nicely balanced in the steady state In the latter there is a disparity between the amount of substrat extracted and that oxidized as shown in TABLE 1 In this experiment cardiac muscle slices from healthy ducklings were incubated in sextuplicate Warburg flasks with pyruvate lactate, and glucose *together* in concentrations usually found in arterial blood One of these three substrates was tagged w th C^{14} in each pair of flasks, so that the production of $C^{14}O_2$ from ea h substrat could be observed in the presence of the other tw The extraction of substrate was measured chemically and the amount of $C^{14}O_2$ captured in the center well over a 20-min. period was determined by end-window counting of $BaC^{14}O_3$ Both the extraction and oxidation of each substrat are expressed in terms of μl of 3-carbon acid metabolized per mg dry weight per hour It may be seen that whereas the total extraction of pyruvat lactate, and glucose by the slices exceeded that observed in intact heart muscle th

Table 1

Extraction and Combustion of Carbohydrate by Cardiac Muscle *In Vitro* and *In Vivo*

Substrate	Substrate concentration		Glucose equivalents (µl metabolized/mg/hr)		
			In vivo	*In vitro*	
	Mg percentage	mM/l	Extraction	Extraction	Combustion
Pyruvate	1.8	0.2	0.5	1.8	0.6
Lactate	18.0	2.0	3.8	5.2	0.5
Glucose	108.0	6.0	4.3	6.8	0.3
Q_{O_2}			27		14
Percentage substrate extracted oxidized			100		10
Percentage oxygen usage to oxidize substrate			96		30

oxidation of the extracted substrate to $C^{14}O_2$ was markedly reduced in the slices. Only pyruvate was burned as rapidly *in vitro* as *in vivo*. Comparisons between the intact heart (postprandial) and the slices indicated that whereas carbohydrate extracted by the intact heart was burned to completion, that extracted by the slices was oxidized to the extent of only 10 per cent. The total rate of oxygen consumption of the slices was about 50 per cent of that of the intact heart, and the combustion of added substrate in the slices accounted for only 30 per cent of this oxygen consumption. It must be concluded from these experiments that although *in vitro* studies of cardiac metabolism may partly reflect the qualitative aspects of cardiac metabolism *in vivo*, there are marked differences in the rates and patterns of metabolism in the two preparations.

Pyruvate is a prime substrate for cardiac muscle both *in vitro* and *in vivo*. In FIGURE 3 are presented the results of an *in vivo* experiment with pyruvate 2-C^{14} in the intact dog in which the apparent half life of blood pyruvate is 1.5 min. Following the intravenous administration of 10 mg./kg. of tagged pyruvate, the coronary sinus blood became rapidly enriched with $C^{14}O_2$ that remained "hotter" than the mixed venous $C^{14}O_2$ for nearly an hour. The dotted line represents the cumulative excretion of radioactivity in the breath that reached 30 per cent of the administered dose in 2 hours. It is estimated that about 20 per cent of the pyruvate oxidized in the first hour was catabolized by the heart.

The state of nutrition of the organism influences markedly the kind of substrate used for energy production by the heart. Under physiological conditions of feeding and fasting the heart shifts from the predominant utilization of carbohydrate to the almost exclusive utilization of fatty acids as a source of energy. Under postprandial conditions or after glucose infusions the myocardium of both dogs and man utilizes mainly glucose, lactate, and pyruvate as sources of energy and the respiratory quotient of the heart approaches 1.0. After an overnight fast, the pattern of metabolism shifts to a greater

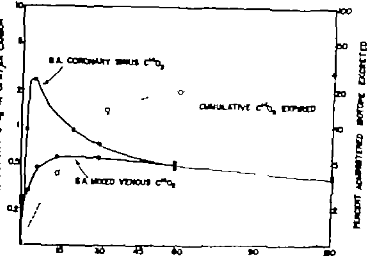


FIGURE 3 The metabolism of pyruvate-2-C^{14} in the intact dog. The solid lines represent the changes in specific activity of carbon dioxide from coronary sinus and mixed venous blood, respectively. The dotted line is the cumulative recovery of radioactivity in the breath as $C^{14}O_2$ expressed (right ordinate) as per cent of administered dose.

dependence upon fatty acids,[17] with a drop in myocardial respiratory quotient to about 0.82. Gordon and Cherkes[1] have recently shown that the source of the fatty acids utilized by the heart is the nonesterified fatty acid fraction (NEFA) bound to serum albumin. In their study of postabsorptive human subjects, it was found that 25 to 70 per cent of the simultaneous oxygen usage of the heart could be accounted for by the uptake of nonesterified fatty acids. These shifts are presented in TABLE 2. With prolonged fasting and with diabetes mellitus, the uptake of carbohydrate by the heart is further depressed with a decrease in the extraction coefficients for glucose, pyruvate and lactate; the myocardial respiratory quotient drops to 0.70 and the heart depends almost solely upon fatty acids for energy.[4]

During transitions in nutritional status from feeding to fasting, the uptake of total carbohydrate and NEFA varies reciprocally and correlates with

TABLE 2
EFFECT OF FEEDING AND FASTING UPON MYOCARDIAL SUBSTRATE UTILIZATION IN DOG AND MAN

Conditions	Fed	Fasted
A-V difference		
O_2 ml per cent	10.80	11.50
CBH mM C 3/l	1.49	0.51
NEFA mM C 16/l	0.01	0.12
Percentage O_2 utilization accounted for by		
CBH	92	30
NEFA	5	58

the observed respiratory quotient for the heart suggesting that these are the major substrates for energy production in heart muscle. Carbohydrate appears to be the preferred substrate if it is available and the animal is in a "fed" state; therefore it appears that arterial level of carbohydrate is the active determinant of the fuel mixture taken up by the heart and that the NEFAs are "passive."

In thiamine deficiency in rats, dogs, and man,[26] the myocardial extraction coefficients for pyruvate and lactate are markedly depressed, the respiratory quotient is reduced, and fatty acids are utilized to an increased extent. There also appears to be reduction in total myocardial oxygen consumption under these conditions and a discordant relationship between myocardial oxygen extraction coefficient and coronary flow. This failure to increase oxygen consumption in proportion to increased work load may be the critical factor in the genesis of the high-output congestive heart failure seen in man with beriberi.

Metabolism of Heart Muscle in Dogs with Thyrotoxicosis and Severe Valvular Disease

Since thyrotoxicosis is a cause of high-output heart failure and valvular disease a cause of low-output failure in man, it was thought of some interest to compare the metabolism of the heart under these two conditions in a suitable experimental animal. The dog was chosen for these studies chiefly because of the large amount of data on cardiac metabolism and hemodynamics available in the literature for this animal under physiological conditions, and because both thyrotoxicosis[2] and congestive heart failure secondary to valvular disease[22] have been produced in this species.

In our study, thyrotoxicosis was produced by feeding healthy dogs 1000 mg/kg/day of U.S.P. thyroid powder for two to four months. Weight loss in the presence of markedly increased caloric intake, markedly increased total oxygen consumption, mild tachycardia, increased excitability, increased rectal temperature, increased hematocrit, and increased levels and turnover rates of protein-bound iodine were noted.[23]

Chronic low-output congestive heart failure characterized by edema, ascites, weakness, reduced exercise tolerance, cardio- and hepatomegaly, and elevation of both right and left ventricular end-diastolic filling pressures was produced in dogs by surgical avulsion of the tricuspid valves (TI) and stenosis of the pulmonary artery (PS).[22] Although initially right-sided, heart failure induced in this way eventually becomes generalized, as indicated by elevation of filling pressures in the left ventricle and a positive inotropic response of both chambers to digitalis. In FIGURE 4 is shown a series of pressure tracings obtained by cardiac catheterization in two chambers (right auricle and right ventricle) and of the heart and three great vessels (coronary sinus, pulmonary artery, and femoral artery) of a typical dog before surgery, after induction of failure, and after treatment of the failure with acetyl strophanthidin, a rapidly acting digitalis drug.[24] Right ventricular end-diastolic pressures, markedly elevated after the onset of failure, were reduced within

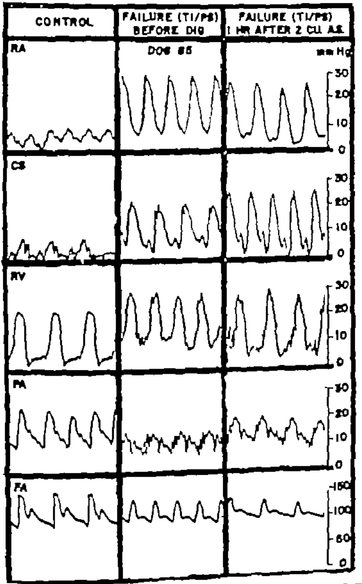


FIGURE 4 Representative pressure tracings in the right auricle, coronary sinus, [illegible] ventricle, pulmonary artery, and femoral artery of [illegible] dog before surgery, after onset [illegible] failure, and after treatment of the heart failure [illegible] cat units of acetyl strophan[illegible] Each series of tracings is [illegible] seconds long.

one hour after administration of 2 cat units of acetyl strophanthidin with an increase in right ventricular systolic pressure and an increase in cardiac output.

The cardiac metabolism of a series of thyrotoxic dogs and of a series of dogs in congestive heart failure due to valvular disease was compared with that for a series of healthy control animals. Cardiac catheterization with intubation of the pulmonary artery and the coronary sinus was performed on each animal under Nembutal-dial-morphine anesthesia. Pressures in the major vessels and the chambers of the right heart were measured electronically and cardiac output was measured by the Fick principle. Coronary flow was determined by the nitrous oxide saturation method. Arteriovenous differences of oxygen, carbon dioxide, glucose, lactate, pyruvate, and nonesterified fatty acids were measured manometrically or chemically on samples of arterial and coronary sinus blood. After cardiac catheterization artificial respiration was initiated, the chest was opened, and the animal sacrificed by excision of the beating heart. Determinations of total acid-soluble phosphorus, true inorganic phosphorus, and adenosine triphosphate were made on quick-frozen samples of ventricular apex by a modification of the barium fractionation technique proposed by Lepage and Umbreit.[34]

Values for resting cardiac output, coronary blood flow, cardiac oxygen usage expressed as ml./min./100 gm. and as Q_{O_2} for normal and hyperthyroid dogs, and for dogs with congestive heart failure secondary to TI/PS are presented in TABLE 3. It may be seen that whereas the cardiac output is decreased in congestive heart failure associated with valvular disease, it is as markedly augmented in experimental thyrotoxicosis in the dog as in man.[35] Coronary blood flow and myocardial oxygen usage were unchanged from normal in congestive heart failure due to valvular disease in the dog, but significantly augmented in thyrotoxicosis. Although Bing[37] reported no increase in the oxygen consumption of the myocardium in man with hyperthyroidism, convincing evidence that coronary flow and total myocardial oxygen consumption are increased in human thyrotoxicosis has been presented by Rowe and his co-workers.[38]

Data on myocardial substrate and oxygen extraction by postabsorptive normal and hyperthyroid dogs and dogs in congestive heart failure due to TI/PS are presented in TABLE 4. It may be seen that the individual arterial levels and arteriovenous differences for glucose, lactate, and NEFA are not

TABLE 3

CARDIAC OUTPUT, CORONARY BLOOD FLOW, AND CARDIAC OXYGEN USAGE BY NORMAL AND HYPERTHYROID DOGS AND BY DOGS IN CONGESTIVE HEART FAILURE

Condition	N	Cardiac output l./min.	Coronary blood flow ml./min./100 gm.	Oxygen A-V ml. per cent	Myocardial O_2 usage ml./min./100 gm.	Q_{O_2}
Normal	7	2.02 ± .12	91 ± 9	12.0 ± 0.7	10.9 ± 1.2	27 ± 3
CHF (TI/PS)	6	1.38 ± .10	98 ± 15	10.5 ± 0.5	10.5 ± 2.0	25 ± 5
Hyperthyroid	6	4.01 ± .37	121 ± 6	14.8 ± 1.6	17.9 ± 1.4	43 ± 3

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one hour after administration of 2 cat units of acetyl strophanthidin with an increase in right ventricular systolic pressure and an increase in cardiac output

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Condition	No	Cardiac output l/min	Coronary blood flow ml/min 100 gm	Oxygen A V ml per cent	Myocardial O_2 usage ml/min/100 gm	Q_{O_2}
Normal	7	2 02 ± 12	91 ± 9	12 0 ± 0 7	10 9 ± 1 2	27 ± 3
CHF (TI/PS)	6	1 38 ± 10	98 ± 15	10 5 ± 0 5	10 5 ± 2 0	25 ± 5
Hyperthyroid	6	4 01 ± 37	121 ± 6	14 8 ± 1 6	17 9 ± 1 4	45 ± 3

valvular disease no changes in CP or ATP levels were noted in the spontaneous failing myocardium of the heart lung preparation. Since CP levels fall in response to failure of energy liberation (anoxia) or energy conservation (DNP) in the heart-lung preparation i t probable that no biochemical disturbance of this type exists in either f ilure due to valvular disease or thyrotoxicosis. On the other hand these data d not give us an insight to the mechanism by which these ailments do use ongesti e heart failure The shift in oxidative metabolism toward the utilization of fatty acids in thyrotoxicosis that also occurs in diabetes, by itself hardly would constitute a sufficient reason for the myocardium to fail Further study of this problem is in progress.

As regards the biochemical lesion in the myocardium of dogs with congestive heart failure due to valvular disease vidence recently obtain d in our laboratory suggests that a biochemical lef t may st in the contractile mechanism itself.[* *] Myosin was isolated from the hearts of animals in failure and suitable controls, rigorously purified and physicochemical characterization accomplished by study of sediment tion onstants, diffusion constants, partial specific volumes, intrinsic iscosities, nd light-scattering behavior of the two preparations From these onstants th molecular weight of cardiac myosin from normal dogs was estimat d t be about 225 000 whereas that from dogs in failure due to valvular disease was found t be of the order of 750,000 The data are consistent with th view that n abnormal stable aggregate of myosin A is formed in cardiac failure nd that this prevents the formation of an cto-myosin with normal ontractile properties These findings are consistent ith the observations that actomyosin preparations from failing heart muscle of dogs[*] and man[*] show certai bnormal properties and reduced contra tility

Summary

Cardiac energetics may be divided int three phases (1) energy liberation, (2) energy conservation, and (3) energy utilization Under normal conditions these cycles mesh t onvert chemical energy to mechanical work t rates of efficiency as high as 40 per ent Glucose, lactate, pyruvate and nonesterified fatty ids supply th bulk of the energy for cardiac muscle, nd the state of nutrition or endocrine balance may shift the substrate mixture extra ted by the myocardium from preponderantly carbohydrate to preponderantly f t Prolonged fasting, untreated diabetes mellitus, and thyrotoxicosis tend to shift th pattern of myocardial oxidation to a greater utilization of fatty acids In congestive heart failure in dogs and man there is no significant alter tion in oxidative metabolism A tudy of the distribution of phosphorus among the high-energy phosphate compounds has revealed a singular lack of d parture from normal n both thyrot xic dogs and dogs congestiv he rt failure The dat suggest that no serious biochemical lesion ist the phase of energy l beration or energy onservation in either thyrot xicosis or low output congestive h art f ilure secondary to lvula dise se Although coll teral dat suggest that an abnormal ty of ardiac myosin exist th animals w th failure due to valvular disease no clear xplanation of th dele terious effects of thyrot xicosis upon myocardial function is pparent The

effects of hypermetabolism and hyperthyroidism upon the assimilation of phosphate-bond energy by the contractile proteins and the effects of the contractile proteins themselves should be further studied

Acknowledgments

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DISCUSSION

E. ELLENBOGEN (*University of Pittsburgh, Pittsburgh, Pa.*): My remarks will concern the characterization of myosin isolated from normal dog hearts and will be limited to those points that hitherto have been often neglected by physical chemists.

First, the heart must be normal by all physiological criteria, such as normal sinus rhythm, normal cardiogram and normal size. The chest of the animal must be opened under standard surgical techniques, respiration must be maintained, and excision must be rapid and so efficient that normal beats continue after the excised heart has been dropped into ice cold water. Even a short terminal ventricular fibrillation will prevent the isolation of normal cardiac myosin. Second, one must go to extreme pains to purify the isolated myosin. So-called crystalline myosin is always contaminated with some actomyosin, and a succession of preparative ultracentrifugations and reprecipitations is necessary for the preparation of myosin that is homogeneous in all respects. As a consequence, the yields of this protein are rather small, of the order of 300 to 400 mg./100 gm. of tissue. Finally, all physicochemical measurements must be carried out to very dilute solutions, in order to make certain that extrapolations to zero protein concentration are meaningful. Failure to do so will result in a set of physicochemical constants that will produce erroneous molecular weights and shape constants.

With these criteria in mind, we have characterized cardiac myosin from normal dog hearts by sedimentation velocity, sedimentation equilibrium, approach to sedimentation equilibrium, viscosity, partial specific volume, free diffusion, sedimentation boundary spreading, and light-scattering measurements. Using our values for s_{20} of 6.12 S, D_{20} of 2.46×10^{-7} sq. cm. sec.$^{-1}$ and $\bar{v}$ of 0.731, a molecular weight of 226,300 is calculated. From sedimentation equilibrium and approach to sedimentation equilibrium experiments a molecular weight of $223,300 \pm 3,600$ is obtained. From the above data, as well as from the limiting viscosity number (also called intrinsic viscosity) of 50 c.g.s. units, the shape of the molecule can be described as a prolate ellipsoid with an axial ratio of 24:1 and a frictional ratio, f/f_0, of 2.14. Using the mercury blue line, the refractive index increment of myosin is 0.206 and the light-scattering molecular weight is 270,000, which agrees well with the other measurements. All of these measurements are consistent with a model for cardiac myosin of a coil 690 Å long and 28 Å thick.

As Olson has indicated, it was found that these physical constants are markedly altered for myosin isolated from dogs in congestive heart failure. These data will be presented in a forthcoming communication.

As a reference standard, rabbit skeletal myosin was prepared in the same way and studied at concentrations of comparable magnitude. Molecular weights calculated from sedimentation and diffusion data and from light scattering data for rabbit skeletal myosin are in agreement at about 450,000.

ELECTRON TRANSPORT IN CARDIAC TISSUE

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Introduction

The normal contractility of cardiac muscle is dependent on a complex series of events beginning with the entry of oxidizable substrates into the cell and ending with the reversible transformation of actomyosin. An interruption or major alteration of the process anywhere along its path may be expected to result in important changes in the efficiency of the work of the heart. Since this series of interdependent and interdigitating biochemical events is obviously enormously complex, for the sake of simplicity in discussing congestive heart disease we may arbitrarily divide it into 5 groups of reactions (SCHEME 1).

(1) The series of enzymatic reactions whereby metabolites are converted into CO_2 and H_2O, in other words, the chemical transformations of the substrate. Whether we are concerned with the burning of carbohydrate or of fat, the majority of these enzymatic steps serve merely to reshape the chemical structure of the metabolite in preparation for the oxidative step during which energy will be liberated. It is logical to examine the oxidative steps in metabolism in more detail not only because they are the ultimately useful steps but because they are of a much greater order of complexity than the nonoxidative steps preceding and following them.

(2) During each oxidative step machinery must be provided to trap the chemical energy liberated during the oxidation and to pass it along with a minimum of loss to another enzymatic system capable of generating ATP.‡ The machinery performing this task is more or less similar in all the individual oxidative steps of metabolism, and it is commonly referred to as the electron transport system. In an organized metabolic system it must make functional connection with the dehydrogenases associated with the substrate on one hand, and it must be intimately linked to the ATP-generating system on the other hand.

(3) The ATP-generating or oxidative-phosphorylation system must then trap the energy liberated during electron transport and utilize it for the synthesis of ATP. ATP, then, may be directly utilized as the primary fuel for muscular contraction and for synthetic processes, or it may be converted to other compounds in equilibrium with it (creatine phosphate and various nucleoside triphosphates) for purposes of storage or for utilization in energy-requiring transformations.

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† Established Investigator of the American Heart Association.

‡ The following abbreviations are used: adenosine diphosphate, ADP; adenosine triphosphate, ATP; British antilewisite, BAL; coenzyme A, CoA; diphosphopyridine nucleotide, DPN; reduced DPN, DPNH; cytochrome c, cyt. c; flavin adenine dinucleotide, FAD; riboflavin 5-phosphate, FMN; reduced FMN, $FMNH_2$; triphosphopyridine nucleotide, TPN; reduced TPN, TPNH.

Scheme 1

(1) Enzymatic degradation of metabolites
(glycolysis, fatty acid oxidation, Krebs cycle)
(2) Energy-yielding oxidative steps
(electron transport)
(3) Formation of ATP
(oxidative phosphorylation)
(4) The contractile process
(actomyosin-ATP system)
(5) Ionic effects

(4) The actomyosin-ATP system then converts the chemical energy of the terminal phosphate bond of ATP into mechanical work.

(5) The latter process appears to be controlled by changes in the concentrations of diffusible ions during the contractile process.

From the viewpoint of the biochemist, any one of these 5 processes may be potential sites of a metabolic disturbance that ultimately expresses itself as a clinically recognizable congestive failure. We have omitted from this list a number of other factors that obviously influence the mechanical work of the heart, since prevailing opinion does not regard these as common causes in the etiology of the disease. Thus, although O_2 supply may be clearly a limiting factor in the entire series of oxidative events, particularly since cardiac muscle tolerates only about one fifth as much O_2 debt as does skeletal muscle, the rate of O_2 utilization in the failing heart may be normal or even higher than in a normal heart. Alterations in the permeability of cardiac cells to metabolites will not be considered either, since Bing's studies[1] of arterial-venous differences in coronary vessels show that the extraction of all important substrates from the circulation is within normal limits in myocardial failure.

Similarly, a failure of nervous control does not seem to be regarded as a primary cause of congestive failure, although experiments on denervated heart (such as heart-lung preparations) point to the importance of nervous control in determining the efficiency of heart beat.

Among the processes listed in SCHEME 1, the third, fourth, and fifth are discussed elsewhere in this monograph. The first topic, the enzymatic steps in the catabolism of substrates, particularly of carbohydrates, has also been discussed. However, it is well to remember that carbohydrate metabolism is not the only important source of energy of mammalian heart. The oxidation of glucose is usually stated to be of minor importance in supplying the energy requirements for ATP generation. In fact, fatty acid and lactate oxidation are the primary sources of energy for cardiac tissue and cardiac glycogen may be regarded as an emergency fuel. TABLE 1, taken from the work of Bing *et al.*, shows that only about 35 per cent of the O_2 uptake of the normal human heart reflects the oxidation of carbohydrate and carbohydrate intermediates.

Space does not permit a detailed discussion of the pathway of fatty acid degradation that has been defined as a result of the outstanding investigations by D. Green, University of Wisconsin, Madison, Wis.; S. Ochoa, New York University College of Medicine, New York, N. Y.; and F. Lynen, Max Planck Institut für Zellchemie, Munich, Germany. However, since fatty acid oxidation is a major source of energy for ATP generation in heart tissue, h

TABLE 1

RELATIVE CONTRIBUTION OF CARBOHYDRATES AND NONCARBOHYDRATES TO TOTAL MYOCARDIAL O_2 USAGE

Carbohydrate	%	Noncarbohydrate	%
Glucose	17 90	Fatty acids	67 0
Pyruvat	0 54	Amino acids	5 6
Lactat	16 46	Ketones	4 3
Total	34 90		76 9

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mention must be made of the sites along the fat catabolism pathway where energy is generated for ATP synthesis Fatty acids are oxidized in the form of their CoA derivatives in a series of 4 steps leading to the formation of a fatty acyl CoA thioester with 2 carbon atoms less than the parent compound and a mole of acetyl CoA

$$RCH_2CH_2COSCoA \rightarrow RCOSCoA + CH_3COSCoA$$

Of the 4 enzymatic steps involved in each of these β-oxidation steps, 2 are oxidative in nature and are probably coupled to ATP-forming systems. The acetyl CoA generated in fatty acid catabolism is then oxidized via the Krebs tricarboxylic acid cycle as is the acetyl CoA generated from carbohydrate oxidation The catabolism of ketone bodies, a supplementary source of energy for heart muscle, also leads directly to the formation of acetyl CoA Thus, while the terminal oxidative steps in carbohydrate and fat metabolism are common, from the standpoint of energetics, fatty acid and carbohydrate oxidation differ in one important respect, namely that in fat oxidation a relatively larger fraction of the energy is released prior to reaching the stage of the tricarboxylic acid cycle

This brings us to the second topic, the electron transport system, the main subject of this paper What follows is intended primarily for the nonspecialists in the field and, therefore I have attempted to keep the discussion on a relatively simple level, minimizing the presentation of original data at the expense of offering very little information with which most enzymologists are not already acquainted

Electron Transport Mechanisms

During the aerobic catabolism of any oxidizable foodstuff at each oxidative step a pair of hydrogens and of electrons are removed from the metabolite The passage of these electrons and hydrogens to O_2 through a series of steps, the electron transport chain, entails the stepwise liberation of energy (SCHEME 2) Each portion of energy liberated as in going from X to Y is linked to the phosphorylation of ADP to form ATP In cardiac tissue the entire process occurs in the sarcosomes.

For the purposes of this discussion we may classify the important mechanisms of electron transport in heart under four major headings (SCHEME 3) As we learn more about these systems, it becomes increasingly apparent that

Scheme 2

$$\begin{array}{l} \downarrow \\ \text{Metabolite A} \\ \Big|-2H \rightarrow X \rightarrow Y \rightarrow Z \rightarrow O_2 \\ \text{Metabolite B} \\ \downarrow \\ \text{Metabolite C} \\ \Big| \; 2H \rightarrow X \rightarrow Y \\ \text{Metabolite D} \end{array}$$

Scheme 3

MECHANISMS OF ELECTRON TRANSPORT IN HEART

(1) Flavoprotein$_1$ → Flavoprotein$_2$ → Cytochromes → O_2
(2) Dehydrogenase → Cytochrome system → O_2
(3) Dehydrogenase → DPN → Cytochrome system → O_2
(4) Dehydrogenase → TPN → DPN → Cytochrome system → O_2

the similarities among them are much more impressive than are the differences.

The first type of reaction represents the oxidation of a substrate by a flavoprotein, which then requires the mediation of a second flavoprotein, the electron transferring flavoprotein (ETF) to link it to the cytochrome system.

The second type includes cytochrome reducing dehydrogenases that are either flavoproteins or metal-flavoproteins and are apparently capable of passing electrons more or less directly on to the cytochrome system without the mediation of a dissociable coenzyme.

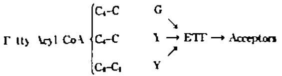
The third type of system starts with the reduction of DPN by one of the DPN-requiring dehydrogenases the DPNH may then be dissociated and passed on to a cytochrome-reducing dehydrogenase system of the second type that accomplishes its reoxidation In some instances the situation may be more complex the reduced DPN may be first required to react with bound sarcosomal DPN prior to joining the cytochrome pathway

The fourth group includes the TPN-reducing dehydrogenases TPNH may be reoxidized in a manner analogous to that of DPNH but there is considerable evidence that in heart in order to link the reoxidation of TPNH to the formation of ATP an altogether different pathway operates that involves a transhydrogenation between DPN and TPNH the DPNH thus formed generates the energy-rich phosphate We shall now examine each of these pathways in detail

Oxidation via ETF The initial step in the dehydrogenation of fatty acid CoA thioesters to the α,β-unsaturated compounds in animal tissues is catalyzed by a group of homologous flavoenzymes (C and Y in Scheme 4), which differ among themselves only in regard to specificity for the chain length of the fatty acid substrate Although the particular enzyme under discussion has been isolated from liver there is little doubt that a similar situation exists in heart In fact, heart mitochondria have been a preferred source for

SCHEME 4

PATHW OF ELECTRON TRANSPORT IN THE DEHYDROGENATION O FATTY ACYL DERIVATIVES OF CoA



From Beinert and Crane reproduced by permission of The Johns Hopkins Press

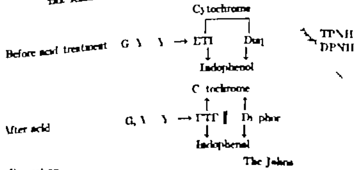
the isolation of many of the enzymes involved in the oxidation of fatty a ids

The peculiar property of the 3 dehydrogenases (G Y and Y') is that they are unable to react with electron acceptors at appreciable rates in the absence of another flavoenzyme ETF whose function is to mediate electron transfer from any of these dehydrogenases to electron acceptors The natural electron acceptor of ETF is not known but in highly purified preparations, reduced ETF is readily oxidized by artificial acceptors such as indophenol dyes, phenazine methosulfate, and ferricyanide more slowly and to a variable extent by cytochrome c and only extremely slowly by O_2 In this last cytochrome c is the only effective natural electron transfer agent but it is doubtful that it accepts electrons *directly* from reduced ETF in intact systems One of the reasons for this is that the interaction of reduced ETF with cytochrome c is very omplex (SCHEME 5) Beinert and Crane who first described this enzyme have found that while highly purified preparations of reduced ETF r rapidly with dyes and considerably more slowly with ytochrome c parations acquire a high activity toward cytochrome c after acid $_2SO_4$ treatment and some further fractionation, but their activity toward dyes remains either unchanged or declines

To make the situation even more complex, the same purified preparations of ETF prior to acid $(NH_4)_2SO_4$ treatment, contain a high diaphorase (DPNH and TPNH dehydrogenase) activity toward dyes but very little ability to

SCHEME 5

SCHEMATIC REPRESENTATION OF TH MUTUAL INTERFERENCE OF ETF AN THE ASSOCI TED DPNH AND TPNH DEHYDROGENASE IN ELECTRON T NSFER TO C TOCHROM AND THE RELEASE OF THIS I TERFERENCE CID TREATMEN



From Beinert an The Johns

link the oxidation of reduced pyridine nucleotides to cytochrome *c*. After acid $(NH_4)_2SO_4$ treatment fractions may be obtained with high ETF and low diaphorase activity and other fractions with high diaphorase and low ETF activity; the latter are also cytochrome reductases. In other words, acid $(NH_4)_2SO_4$ treatment permits the separations of ETF and DPNH oxidizing activities to a large extent and increases the ability of both types of enzymes to react with cytochrome *c*. Mixing of the two preparations re-establishes conditions similar to those that existed prior to acid $(NH_4)_2SO_4$ treatment, that is, inhibition of cytochrome reduction by either ETFH₂ or by reduced pyridine nucleotides. It seems that in the original untreated enzyme the oxidation of DPNH and of fatty acid via ETF by cytochrome *c* are mutually inhibitory. On further investigation this might prove to be some type of control mechanism that may have considerable importance in tissues where energy generation is largely dependent on fatty acid metabolism.

The cytochrome-reducing dehydrogenases. For many years a group of oxidizing enzymes known to pass electrons to the cytochrome system without the benefit of mediation by mobile soluble co-enzymes have been referred to as cytochrome reducing dehydrogenases. In heart the most important members of this group are the enzymes responsible for the oxidation of succinate and of DPNH. Because of the key position of the former in the Krebs cycle and of the latter in coupling pyridine-nucleotide dehydrogenases to O_2, by far the majority of energy generation in cardiac tissue involves the participation of these two enzyme systems. In fact, in all likelihood we are not dealing with two enzyme systems, but with one structural and functional unit interlinking somewhere near the level of cytochrome *b*, which is called the electron transport chain (scheme 6).

Mammalian heart has always been the favorite material for the study of this chain of enzymes, and much of what we know has been learned from experiments with heart preparations. In heart sarcosomes and even in submitochondrial units derived from them, the electron transport chain behaves as a highly organized system of enzymes with a definite structure. In fact, for many years it was believed that structural organization plays such an important role in the activity of the system that purification and isolation of its individual enzymes would be impossible. Although, until a few years ago, attempts to isolate the components of the system met with uniform failure, much was learned about the properties of the whole from spectroscopic studies and from kinetic experiments on the oxidation of succinate and DPNH. The components listed in scheme 6 have been recognized on the basis of their

SCHEME 6

SCHEMATIC REPRESENTATION OF THE ELECTRON TRANSPORT CHAIN

Succ → Iron flavoprotein (S.D.) ↘

Cyt *b* → Cyt → Cyt → Cyt → Cyt a_3 → O_2

DPNH → Iron Flavoprotein (DPNH-D) ↗

spectra in such particulate preparations. In addition to the hemoproteins listed the existence of a factor acting between b and c_1 has been repeatedly proposed on the basis of the inhibition of the chain by BAL, antimycin,[7] and certain naphthoquinones, which were thought to combine with it however direct proof of the existence of this factor has not yet been found.

We shall now briefly survey the salient properties of this chain starting with the primary dehydrogenase Succinic dehydrogenase, isolated from heart as a soluble protein, contains one mole of flavin and four atoms of Fe as prosthetic groups.[8] The flavin moiety (FAD) is unique among flavoproteins in that it is joined to the protein in such strong linkages that it cannot be separated from the bulk of the protein except by proteolysis of the latter even then it does not come off as FAD The participation of this flavin in the catalysis is unambiguous it is the primary oxidant of succinate and the reductant of fumarate The four nonhemin Fe s are more loosely attached since they are set free upon denaturation of the protein Their role in the catalytic activity is still uncertain At least two of the four Fe atoms are essential for the activity of the soluble enzyme, but despite extensive investigations no evidence has been found to support the possibility that they undergo oxidoreduction [10]

The catalytic properties of the dehydrogenase are quantitatively the same in the isolated highly purified state as in sarcosomal preparations the enzyme is inhibited by malonate and other substrate analogues but not by antimycin or BAL it occurs in a form of low catalytic activity and may be activated many times by contact with succinate or with analogues of the substrate [11]

In one respect the soluble enzyme behaves very differently from its counterpart in particulate heart preparations upon solubilization the reactivity toward many electron carriers is lost altogether and it is greatly reduced toward others This is largely due to the separation and removal of other reaction sites (that is, other proteins) by the solubilization procedure The relative reaction rates of the dehydrogenase in a typical particulate preparation of the organized respiratory chain from heart are shown in TABLE 2. Phenazine methosulfate measures the full activity oxidation via the electron transport chain, supplemented by externally added cytochrome c measures most of the activity dyes like methylene blue measure only a fraction of the activity and that only

TABLE 2

ACTIVITIES OF KEILIN-HARTREE PREPARATION WITH VARIOUS ACCEPTORS AT 38° C (V_{max} VALUES)

Assay	μmoles substrate removed/mg/hr	Per cent of max activity*
Phenazine methosulfate	55.2	100
O_2	23.8	43
O_2 (with added cytochrome c)	52.8	96
Ferricyanide	29.2	53
Methylene blue	13.8	25
Brilliant cresyl blue	3.8	6.9
$FMNH_2$-hydrogenase-H_2	2.3	4.2

* Phenazine methosulfate taken as 100

TABLE 3

RELATIVE REACTION RATES OF BEEF HEART SUCCINIC DEHYDROGENASE AT 25° C.

Acceptor or donor	Rate: Su → F	Rate: F → Su	Remarks
O_2	0.02	0	
Ferricyanide	39	0	
Cytochrome c	0.01 (approx.)	0	
2,6-Dichlorophenolindophenol	0.6		
Phenazine methosulfate	100	2 (approx.)	
Phenazine ethosulfate	100		
Brilliant cresyl blue	0.7		
Methylene blue	2	2.5	
Pyocyanine	3	33	
2-Hydroxynaphthoquinone	0	+	V_{max} not measured for F → Su
FMN	0	11	
FAD	0	++	V_{max} not measured
Diethylsafranin	0	11	
DPN	0	0.1 (approx.)	V_{max} not measured
Benzyl viologen	0	3	
Phenazine α-carboxylate	0	0	

All the rates are compared with the V_{max} for phenazine methosulfate in the forward direction (6 μmoles of succinate oxidized per min per mg of enzyme in 0.05 M phosphate, pH 7.6, 25° C.), which is arbitrarily taken as 100, whether the reaction is measured in the direction of fumarate reduction or of succinate oxidation. The signs + and ++ indicate slow and fast rates that have not been measured quantitatively. All the rates were determined in the presence of 0.02 M succinate or 0.033 M fumarate.

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at extrapolated infinite dye concentration. At actual concentrations the activity may be much lower. Ferricyanide measures only about one half of the activity of the enzyme and, finally, the reverse reaction, the reduction of fumarate to succinate with FMNH₂ as electron donor, proceeds at less than one twentieth of the rate of the oxidation of succinate.

When we compare these activities with similar data for the soluble dehydrogenase in an essentially homogeneous state (TABLE 3) some striking differences become evident. As expected from the fact that the cytochrome chain has been removed, the reactions with O_2 and cytochrome c have disappeared, and those with dyes (such as neotetrazolium) that react far along the cytochrome chain, are also lost. Since in particulate preparations ferricyanide has at least two reaction sites,[38] the chief one being at the level of cytochrome c, and the lesser one with the dehydrogenase, the isolated dehydrogenase reacts relatively little with ferricyanide. The reaction of methylene blue and brilliant cresyl blue, dyes that once served as the routine ways of the dehydrogenase in tissues, with the dehydrogenase is also negligible and we may surmise therefore that they react somewhere above the level of the dehydrogenase. In the reverse reaction, electron donors, which react directly with the FAD moiety of the dehydrogenase, retain their full activity on solubilization and isolation of the enzyme. The most surprising fact in this table

is that, as compared with $FMNH_2$ as electron donor even the activity with phenazine methosulfate had been reduced to about one half after isolation of the enzyme. As we now know this is because phenazine methosulfate also has two reaction sites in the transport chain, one with the dehydrogenase as we know it and one slightly farther along the chain the latter is lost when the dehydrogenase is isolated It may be of interest to illustrate some of the techniques by which such conclusions may be reached

In mitochondria the oxidation of succinate by neotetrazolium and related dyes is antimycin-sensitive hence their reaction site should be above the level of cytochrome *b* In fact there is some evidence that cytochrome oxidase is the site of the reaction Similarly in such a system a major part of the succinate-ferricyanide reaction is inhibited by antimycin thus it would clearly be expected that a major part of the succinate-ferricyanide reaction would be lost in separating succinic dehydrogenase from the cytochrome chain This is, in fact, the case

Another technique is to compare various types of preparations of the electron transport chain and of fragments thereof all of them capable of oxidizing succinate at high rates, with regard to their relative reactivities with various electron acceptors The various types of particulate succinic oxidase and succinic dehydrogenase preparations in TABLE 4 have been claimed to have very different dye specificities, one of them reacting only with brilliant cresyl blue but not with methylene blue, others only with such substances as indophenol dyes Such contentions seemed unlikely since the reaction sites in the cytochrome chain could hardly have equaled in number the different preparations claimed to have singular individual specificities. In fact, each group of investigators had its own favorite preparation endowed with a special name and for those not working directly in the field, the situation must have seemed somewhat chaotic. It appeared quite probable that many of the differences were reflections of the different assay techniques used by individual workers In fact, most investigators assayed their preparations at a single arbitrary dye concentration which may have been so low for one dye that it

TABLE 4
RELATIVE ACTIVITIES OF VARIOUS PARTICULATE PREPARATIONS FROM ANIMAL TISSUES WITH DIFFERENT CARRIERS

Type of preparation	Relative maximal activity (V_{max})				
	Phenazine methosulfate	Methylene blue	Brilliant cresyl blue	Ferricyanide	Indophenol
Keilin Hartree	100	24	9	54	15
Ball Cooper succinic oxidase	100	16	6	48	28
Ball Cooper deoxycholate extract	100	17	8	50	15
Green "SDC"	100	23	10	72	10
Stotz *et al* prep A (cholate treated)	100	20	8	69	39

All activities are V_{max} values at 38° C *p*H 7 6

measured only a fraction of t tivity and so high for nother dye that the enzyme was almost complet ly h bited by it. As we have discussed else here, reliable turnover t th oxidizing enzymes may be derived only from maximal activities at polated infinite dye concentration based on precise kinetic data.

The nzyme preparations l t l TABLE 4 include some that possess the complete electron transport l some that contain only cytochrome b and c and some that although t ll t late are largely devoid of cytochromes In our laboratory Grudit f l F t by taking the activity with phenazine methosulfate as 100 the r t th dyes whose reaction site is close to the dehydrogenase (meth l l l rilliant cresyl blue $FMNH_2$) is the same in all the preparation W th ptors such as cytochrome c or neotetrazolium, the activity would r m large figure to 0 and with electron carriers such as ferricyanide n l t l d the activity drops off in preparations in which the cytochrome h l t From this type of data and from our knowledge of the tochrome ont t of the different preparations we may deduce which electron arriers act lose to the d hydrogenase and which have reaction sites farther removed from it

A very different technique for the exploration of reaction sites in the electron transport chain is the use of differential inactivation. Low concentrations of cyanide of course insta taneously inhibit cytochrome oxidase As is known from the work of Tsou higher oncentrations of cyanide more slowly inactivate the oxidation of succinat with ertain acceptors in succinic oxidase preparations As a result of systematic study of this phenomenon, Grudittа[14] has shown that when th cyanide ina tivation is omplete 0 of the reactivity of the dehydrogenase with methylene blue and brilliant cresyl blue is lost but only 50 per cent of the tivity with phenazine methosulfate and about 70 per cent of the activity with ferricyanide (corresponding to the antimycin-sensitive portion) are bolished In other words, that part of the activity of an electron carrier that reflects direct reaction with the dehydrogenase is fully retained As expected the cyanide-inactivated enzyme still reacts maximally with FMNH Thus inactivation by cyanide converts a highly organized respiratory system t one that beha es much like the isolated primary dehydrogenase

One reason for emphasizing this phase of the ork on the succinic oxidase system is t point out some of its omplexities and the consequent danger in deriving conclusions bout the activity of the system in normal or pathological samples without due onsideration of the dequacy of the assay method Differential inactivations and inhib tions in one assay but not in another are frequently encountered in work w th the electron transport system Such effects often occur "spontaneously" and may lead t artifact tious results[16]

There is another point of particular interest t biologists In animal tissues whose physiological functioning depends on continuous supply of energy derived from erobic processes, as in heart the properties of succinic dehydrogenase meet this requirement excellently in that the forward or energy yielding reaction (succinat oxidation) predominates by far over the reverse

TABLE 5

REACTION VELOCITIES OF BACTERIAL SUCCINIC DEHYDROGENASE WITH VARIOUS CARRIERS

Electron carrier	Relative rate (V_{max})		
	M. lactilyticus	*P. pentosaceum*	*Proteus vulgaris* OX1
Phenazine methosulfate	100	100	100
Methylene blue	88	62	Low
Leucomethyl viologen	3900	33	900
$FMNH_2$	325	33	134
Ferricyanide	152	—	—
Ratio $\frac{Su \rightarrow F}{F \rightarrow Su}$	1/39	3/1	1/9

Unpublished data of F. J. S. Lara, M. G. P. J. Warringa, and T. P. Singer.

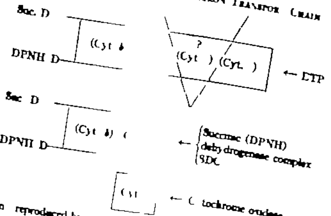
or reductive, reaction. The same is true of other cells with similar metabolic requirements, as, for example, aerobic yeasts. In contrast, in obligate anaerobes the enzyme catalyzes the reductive reaction about thirty times faster than the oxidative one in the pure state; in whole cells its ability to oxidize succinate is further depressed by the presence of an inhibitor that selectively inhibits succinate oxidation without affecting fumarate reduction, so that fumarate reduction predominates one hundred times over succinate oxidation. In facultative anaerobes, such as *Propionibacteria* and *P. vulgaris*, which require the oxidative process under aerobic conditions and must also be able to reduce fumarate under anaerobic conditions, the properties of the enzyme are again excellently suited to the physiological needs, since the forward and reverse reactions function approximately equally well (TABLE 5).

Turning to the rest of the electron transport system, we find that the information on its other components is considerably more limited. Only cytochrome *c* has been isolated in pure state from animal tissues. Cytochrome *b* type proteins have been obtained in highly purified soluble form from microorganisms[39] and from liver microsomes,[21, 22] but the isolation of the classical cytochrome *b* of the respiratory chain of animal tissues has not yet been accomplished. Like cytochrome *b* from heart, cytochromes c_1 and *a* and a_3 (the latter two together known as cytochrome oxidase) have been obtained only as partially purified particle fractions or as bile salt preparations. In view of the lack of highly purified preparations and of specific assays for the measurement of the catalytic activities, it is not surprising that information on their chemistry and properties is very limited.

With the advent of methods for the purification of submitochondrial particles and of their lipoprotein constituents, recent years have witnessed considerable success in the isolation of various fragments of the cytochrome chain in several laboratories. SCHEME 7, taken from Green and his associates, is a diagrammatic representation of what happens when a preparation of the entire

Since this review was written, Järnefelt *et al.* reported the isolation of cytochrome *a* as a soluble lipoprotein[38a].

Scheme 7



From Green reproduced by permission of the Academic Press Inc

electron transport hain, here denoted a ETP is exposed to the action of deoxycholate or holate in the presenc of isobutanol or trypsin The oxidase chain is broken into two fragments, a green particle with cytochrome oxidase activity and a red one here referred t a SDC The latter contains only cytochromes *b* and *c* (here referred to by t earlier name cytochrome) This latter fragment react with ytochrome but not with O_2 It can be degraded further by desiccation with acetone followed by alkaline extraction to yield an active preparation of succinic dehydrogenase and an inactive hemoprotein residue containing cytochromes *b* and

In addition to hemoproteins, purified preparations of the electron transport system contain considerable amounts of non-hemin metal ions (Fe and Cu) and as much as 40–60 per ent lipids Among the lipids a vast number of individual compounds have been recognized ranging from neutral fats to fat-soluble vitamins

A number of years ago i as suggested by Ball and Cooper[20] that the function of the lipids is to cement the individual enzymes in the hain to each other in a highly specific onfiguration In more recent times som more specific additional functions have been proposed Thus Nason and Lehmann[20] have found that in particular preparations from animal tissues the flow of electrons between succinate and cytochrome and between DPNH and cytochrome is interrupted when the enzyme preparation is repeatedly extracted with suitable organic solvent, such as iso-octane The preparation could be reactivated by the residue obtained upon concentration of the extracting solvent and by pure vitamin E Nason and Lehmann also showed that the extraction removes certain amount of vitamin E from the preparation They suggested that vitamin E acts as a cofactor possibly directly undergoing oxidation-reduction, between cytochromes *b* nd Later these workers, as well as other investigators,[22] found that reactiv tion of the

TABLE 6
EFFECT OF Q 275 ON SUCCINOXIDASE

Enzyme treatment	Additions	Activity µl atoms oxygen 5 min mg
None		2 3
None	Q 275, 0 088 mg	2 5
None	Cytochrome 1 mg	2 6
Extracted		0 4
Extracted	Q 275, 0 088 mg	2 7
Extracted	Cytochrome 1 mg	2 9

From Crane *et al* [87] reproduced by permission of the W B Saunders Company

extracted preparation could be even more effectively accomplished by a variety of neutral fats and other lipids, including oleomargarine Because of the unspecificity of this reactivation and the fact that only about 10 per cent of the vitamin E content of the preparations was removed by solvent extraction resulting in almost complete inactivation,[29] Slater and others have questioned the role of vitamin E in the electron transport system [33] The idea nevertheless deserves serious attention but the phenomenon is highly complex, as is everything else in this field, and its conclusive demonstration is fraught with experimental difficulties Thus, more recently Donaldson and Nason[34] have been able to show that in certain aged preparations reactivation of solvent-extracted samples is brought about quite specifically by vitamin E and suggested that the explanation for the unspecific reactivations observed may be that various lipids move the vitamin E still remaining in extracted samples to the active site where it then functions

Among the lipid compounds extracted from heart sarcosomes by iso-octane and similar solvents is a quinone of as yet undetermined structure to which its discoverers, Crane *et al* [87] refer as Q-275 The quinone is said to be much more concentrated in purified preparations of particulate succinic dehydrogenase than in the original sarcosomes. Preparations of the electron transport chain are capable of oxidizing and reducing it this, in itself, is not a good criterion of physiological activity but more significantly it has been reported to be isolable in the *oxidized* form when the preparations are extracted after exposure to air but in the *reduced* form when they are extracted following exposure to cyanide and succinate As seen in TABLE 6, heart preparations in which succinic oxidase activity has been abolished by solvent extraction are fully reactivated by a small amount of Q-275 but not by other fats tested, including vitamin E. The interpretation of their observations is again complicated by the fact that besides the quinone cytochrome *c* also reactivates at low levels, despite the fact that cytochrome *c* is not extracted by the solvents used however the interest of the observations is undeniable

Another highly interesting observation whose full significance cannot be assessed at this time has been reported recently from the same laboratory [88]

Q 275 (mitoquinone) is apparently identical[88a] with ubiquinone previously isolated in Morton's laboratory [88b] For more recent studies of its metabolic role see references [88c]

Table 7

T Restoration of DPNH Oxid Activity Iso oct r Extracted PTP

Number of extractions	DPNH oxidase				DPNH cytochrome reductase	DPNH ferricyanide
	No addition	+ Cytochrome	Lipid alone	Lipid cyto-chrome		
None	1 2	1 3	1 3	1 2	0 10	2 5
One	0 2	1 8	0 1	2 0	0 35	5 9
Three	0 0	0 35	0 0	1 9	0 50	2 5

Three tenths mg phospholipid fraction in ethanol or 0 02 ml 1 per cent cytochrome C added as indicated DPNH oxidase was ithout emene All activities expressed as moles DPNH oxidized/min mg protein

From Widmer and Crane [*] reproduced by permission of *Biochimic et Biophysica Acta*

According to this report following alcohol treatment of sarcosomes a lipid-bound cytochrome that is soluble in nonpolar solvents but not in water may be extracted by iso-octane The sam lipid-cytochrome is formed from ordinary cytochrome upon add tion of a phospholipid fra tion isolated from the sarcosomes Whereas solvent-e tracted succinic oxidase prepara tions, as just shown are fully rea tivated by either Q- 75 or ordinary cytochrome c in regard to the ur mat cytochrome reaction the DPNH-oxidizing ability of the ame samples is only slightly restored by ord nary ytochrome but is fully reactivated by lipid ytochrome c (table 7) The puzzling aspect of this report is that above ytochrome b where this factor is presumed to act, the pathway of electron transport is generally thought to be a common one for succinate and DPNH oxidation

Before leaving the subject mention should be made of the role of itamin K in the chain as postulated by Martius and Nitz Litzow[*] (scheme 8) who state that vitamin K would act bet een DPNH and cytochrome b nd thus would not play a direct role in succinat oxidation however they onsider vitamin K to be on the normal pathway for DPNH oxidation when the latter is coupled to the generation of ATP while the ytochrome reductase pathway would be a nonphosphorylating bypass

The evidenc on which this scheme rests is indirect, and attempts to repeat some of th key observations have be n unsuccessful Colpa Boonstra and Slater[*] ha shown that itamin K_1 is act vely oxidized and reduced by re-

Scheme 8

Role of Vitamin K the Electron Transport Chain According to Martius Nitz Litzow[*] Modified Colp Boonstra nd Slater[*]

Succ dehydrogenase ⇄ succinate

⇅

Substrat → DPN → t k → cı b → factor → Cy →

↘ ↗

flavoprotein

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spiratory chain preparations with attendant oxidative phosphorylation. This, in itself, is only compatible with a physiological role but in no way proves it, since a large number of substances unrelated to physiological processes behave similarly. An active participation of vitamin K in the chain remains a possibility, but no good evidence has as yet been found to support it.

As may be surmised from the foregoing, investigations of the constitution and mechanism of action of the electron transport system are in a vigorous state with a multiplicity of unexplained observations awaiting exploration. I feel that clear-cut answers to many of these problems will have to await the isolation of the individual enzymatic components in a high state of purity, the development of specific assays for each, and possibly the reconstruction of the whole from its components.

DPNH oxidation. In heart the oxidation of DPNH, like that of succinate, is associated with sarcosomes and is mediated by a "cytochrome reducing" dehydrogenase. Certain important differences exist, however, between the events in succinate and in DPNH oxidation.

While the initial reaction on the succinate path is an unambiguous one between succinate and its dehydrogenase, the initial events in DPNH oxidation are more complex. About 20 years ago Straub[illegible] isolated from heart a flavoprotein, DPNH diaphorase, which catalyzed the oxidation of DPNH by dyes but not by cytochromes. About 10 years later Mahler *et al.*[illegible] isolated another mitochondrial flavoprotein from the same source, DPNH-cytochrome reductase, which catalyzed DPNH oxidation by cytochrome *c*. This enzyme, while possessing diaphorase activity, was distinguished from Straub's diaphorase by virtue of a much higher nonhemin iron content (4 atoms per mole, as in succinic dehydrogenase). Some years later Mahler and Elowe[illegible] proposed, on the basis of some indirect but clever experiments, that the iron components in the reductase participate in electron transport to cytochromes and that the diaphorase, being largely devoid of these, cannot reduce cytochromes and is therefore a degradation product of the reductase. Subsequent experiments by Massey[illegible] removed the experimental basis for assuming that iron has an oxidoreductive role in this enzyme (although this still remains a possibility). The iron nevertheless appears necessary for the *binding* of cytochrome *c* by the soluble reductase, but the physiological significance of this fact is uncertain since, in the electron transport chain, not cytochrome *c* but cytochrome c_1 (or possibly *b*) is the first hemoprotein to be reduced by DPNH.

The contention that diaphorase is an artifact of the isolation procedure, thus a degraded cytochrome reductase, is weak since (1) the procedures for the solubilization of both enzymes from sarcosomes are drastic and very similar, (2) diaphorase has been shown to exist in intact circulating reticulocytes,[illegible] and (3) recently an important physiological function has been proposed[illegible] for diaphorase.*

Before discussing this function we must consider how DPNH generated by pyridine nucleotide enzymes inside and outside heart mitochondria gets to

* Very recently Massey[illegible] has demonstrated conclusively that diaphorase is not [illegible] degraded cytochrome reductase. He has shown also that diaphorase acts as [illegible] very potent lipoic acid dehydrogenase.

the dehydrogenase, which reoxidizes it and thus starts off the chain of events in energy generation. One possibility is that DPNH, being in most cases a dissociable mobile coenzyme, comes off the pyridine nucleotide dehydrogenase and somehow reaches the structurally bound DPNH oxidizing system of the sarcosomes. A moment's reflection brings to mind some difficulties with this mechanism. Externally added DPNH is certainly oxidized by heart mitochondria with consequent generation of ATP. As has been known for many years, mitochondria contain considerable quantities of tightly bound DPN that cannot be removed by repeated washing.[88] According to Lester *et al.*[89] in heart mitochondria only that part of DPNH oxidation, which is initiated by way of bound DPN, is coupled to oxidative phosphorylation. If this is the case, bound DPN might serve as a link between the electron transport system and the dehydrogenases acting at some distance from it. A link between free and bound DPN is provided by the observations of Weber and Kaplan[90] that several flavoenzymes, notably heart diaphorase, catalyze oxidoreductions between DPNH and its analogues, and these workers have, in fact, suggested that electron transport between pyridine nucleotides might be a physiological function of diaphorase.

Mitochondria and more or less intact preparations of the electron transport chain avidly oxidize DPNH with O_2 as acceptor, but cytochrome c, a proved component of the chain, when externally added, taps off electrons only slightly in such a system and its reduced form is similarly only slowly oxidized (TABLE 8).

Preparations with such characteristics have been termed "closed" by Green and his co-workers,[91] as opposed to "open" DPNH oxidase preparations in which O_2 is a poor, and ferricytochrome *c* a good, acceptor from DPNH and in which ferrocytochrome is rapidly oxidized by O_2. The terminology is meant to imply that the "opening phenomenon" interrupts a closed conductor system as if a wire were cut. Opening of a closed preparation is readily brought about by deoxycholate or trypsin treatment or by $(NH_4)_2SO_4$ and cholate. The latter treatment, in fact, has been shown to cause separation of the particle

TABLE 8‡

TRANSFORMATION OF CATALYTIC PROPERTIES OF DPNH OXIDASE BY DEOXYCHOLATE

Specific activity (ΔO.D./mg. enzyme/min. at 23° C.)

	DPNH as substrate		Reduced cytochrome as substrate
	O_2 as acceptor*	Reduced cytochrome as acceptor†	O_2 as acceptor†
Enzyme	5.6	1.6	1.6
Enzyme + deoxycholate	0.7	26.0	40.0

* Measurements made at 340 mμ.
† Measurements made at 550 mμ.
‡ From Green *et al.*[91] reproduced by permission of *Biochimica et Biophysica Acta*.

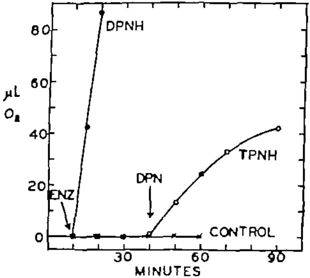


FIGURE 1. The oxidation of DPNH and TPNH. Each experimental Warburg vessel contained, at the start, 2.0 ml. of 0.1 M PO_4 buffer, pH 7.4, 0.1 ml. of cytochrome c, 6 × 10^{-4} M, 8 mg. (approximately 10 μmoles) of DPNH or TPNH, and H_2O. After 10 min. 0.1 ml. of heart muscle preparation was tipped from one side arm. To the vessel containing TPNH, 0.5 mg. of DPN (in 0.1 ml. of H_2O) was tipped from a second side arm after 40 min. The final volume in each vessel was 3.0 ml. The control vessel contained no TPNH or DPNH, but all other components, including 0.5 mg. of DPN. From Ball and Cooper.

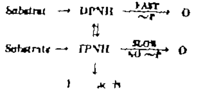
into two derivatives, one with the characteristics of cytochrome oxidase and the other with some of the properties of DPNH-cytochrome reductase in that it catalyzes the oxidation of DPNH by cytochrome c.[48] There are two outstanding differences between the cytochrome reducing particle obtained by this fragmentation and Mahler's purified DPNH-cytochrome reductase. The latter is devoid of hemoproteins and is antimycin-insensitive; the former contains hemins, which are reduced by *DPNH*, and the oxidation of *DPNH* by this preparation is antimycin-sensitive.

Are we then to assume that there are two separate pathways for DPNH oxidation in sarcosomes, the antimycin-sensitive electron transport chain and the DPNH cytochrome reductase or, even worse, that the latter is another artifact of isolation? Not at all. It is well to recall that the ability to reduce cytochrome C rapidly is a basic chemical feature of flavins, retained and accentuated in the flavoprotein DPNH-cytochrome reductase. The reductase may well be the initial dehydrogenase in the chain of DPNH oxidation, passing electrons on through hemoprotein(s) to cytochrome c. When solubilized and isolated from the rest of the cytochrome chain it may still retain its ability to reduce the cytochrome component with which it normally reacts (this point has never been tested since pure preparations of c_1 have not been obtained) but because of a structural similarity in the active center of hemoproteins, it can *also* bind and react with cytochrome c.

TPNH oxidation. The last subject I should like to touch upon briefly is

SCHEME 9

OXIDOREDUCTION OF TPN IN ANIMAL TISSUES



From Kaplan *et al.* reproduced by permission of *Biochimica et Biophysica Acta*

the mechanism of TPNH oxidation. Its importance to cardiac energy generation lies in the fact that one of the Krebs cycle enzymes is TPN-specific and TPN is also the prosthetic group of other dehydrogenases of importance in heart metabolism. The oxidative phosphorylation accompanying TPNH oxidation in heart mitochondria is said to be much less extensive than is the case with DPNH.[a] In fact partially purified preparations of the electron transport system from heart cannot even oxidize TPNH. A clue to the possible mechanism of TPNH oxidation and accompanying phosphorylation in heart comes from the work of Kaplan[a] and his colleagues who have demonstrated that the enzyme pyridine nucleotide transhydrogenase which catalyzes oxidoreductions between pyridine nucleotide coenzymes, is present in mammalian mitochondria. FIGURE 1 reproduced from Bell and Cooper[a] provides evidence for the role of the transhydrogenase reaction in TPNH oxidation by preparations of the electron transport system of heart. The preparation oxidizes DPNH but not TPNH and a catalytic amount of DPN initiates the oxidation of TPNH. SCHEME 9 presents Kaplan's ideas of the sequence of events in oxidoreductions of TPN in animal tissues. The TPN reduced by dehydrogenases interacts with DPN via transhydrogenase regenerating TPN and forming DPNH. The latter is then oxidized via the DPNH oxidase pathway with the concomitant generation of ATP. A part of the TPNH escapes this route and serves in reductive processes, such as fatty acid synthesis. Thus transhydrogenase would assume a possible role here as a potential regulator of TPNH metabolism.

Concluding Remarks

The foregoing description, even if sketchy and perhaps oversimplified, describes the state of our knowledge of electron transport mechanisms in heart. As may be seen, the problems are complex and the experimental difficulties considerable. It is well to be aware of the uncertainties, difficulties, and many pitfalls in the field, particularly if one is to embark on an investigation of pathological changes in the system.

The electron transport system is like a highway with relatively few roads of access but with many branches that lead off it. Some of these branches are bypasses in that oxidation occurs without any conservation of energy. Clearly there are many ways in which the main path may be blocked. Thus the demonstration of the presence of the component enzymes in pathol-

sample is no indication of the functioning of the over-all system. Furthermore, a measurement of the rate of removal of oxidizable substrates, and even of total O_2 uptake, gives no indication of how much of the oxidative energy was conserved in the form of ATP. The possibilities of metabolic derangements are numerous; many of them may lead to uncoupling of oxidation and phosphorylation.

From the standpoint of localizing pathological changes, the efficiency of the over-all functioning of the energy-generating system is open to and ripe for systematic study, even though the intricate details of the machinery are not yet clearly defined. A systematic comparison of the rates of oxidation of some key metabolites and of the efficiency of the accompanying phosphorylations in sarcosomes from normal and pathological hearts seems to be highly desirable.

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OXIDATIVE PHOSPHORYLATION IN MUSCLE MITOCHONDRIA AND ITS POSSIBLE REGULATION

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As my work has been done on liver mitochondria rather than on heart muscle, it becomes incumbent upon me to compare the work on liver mitochondria with that reported on heart mitochondria, also called sarcosomes. Oxidative phosphorylation per se will not be covered as, at present, the subject is too abstruse and controversial, and the mechanisms are still sought. I shall emphasize the regulation of oxidation through the coupled oxidative phosphorylation system, a process well known in liver mitochondria and somewhat less well known in heart muscle mitochondria, and consider how this regulation might be reflected in the work of the heart.

First I shall discuss briefly the known rudiments of phosphorylation as coupled to substrate oxidation and to electron transport. The sequence of reactions in the respiratory chain, as given below, is known chiefly through the work of Chance and Williams, while the positions in this chain where ATP is formed are known primarily through the experiments of Lehninger.

$$\begin{array}{l} \text{Substrate} \rightarrow \text{DPNH} \rightarrow \text{flavoprotein} \rightarrow \\ \quad | \leftarrow \text{ATP} \rightarrow | \longleftarrow 2\,\text{ATP} \longrightarrow \\ \qquad\qquad\qquad \text{Cyt } b \rightarrow \text{Cyt } c \rightarrow \text{Cyt } a \rightarrow \text{Cyt } a_3 \rightarrow O_2 \\ \qquad\qquad\qquad \longrightarrow | \longleftarrow \text{ATP} \longrightarrow | \end{array} \tag{1}$$

In the case of some substrates such as glutamate or succinate there is no phosphorylation at the substrate → DPNH step. It is definitely known that the oxidation of reduced cytochrome *c* by oxygen gives one phosphorylation while the oxidation of reduced DPN by cytochrome *c* gives two phosphorylations, one probably between DPNH and flavoprotein and the other probably between cytochromes *b* and *c*. In all cases where the pyridine nucleotides participate in the electron transport chain, three phosphorylations occur during the passage of electrons toward oxygen.

This is all that was known about coupled phosphorylation. Recent discoveries, however, have given us more insight into these reactions. It was found that if inorganic phosphate was labeled with P^{32} and there was ATP in the medium with the mitochondria, an exchange of the radioactivity between the inorganic phosphate and the esterified high-energy phosphate of ATP resulted; this occurred in the absence of substrate. Furthermore ADP labeled in its purine moiety with C^{14} gave an exchange reaction with ATP which then became labeled in its purine moiety. Since these reactions are reversible and since no net electron transport occurs, it was postulated[1] that there exists within the mitochondria a high energy compound that results when ATP reacts with a postulated unknown component. The reactions below give an adequate up-to-the-moment explanation of the recent experimental findings, with $P \sim X$ being the postulated intermediate.

$$ATP + P \quad ADI + P \sim X \tag{2}$$

It is thought that P ~ X reacts with some m mber(s) of the electron transport chain above according to

$$P \sim X + A \quad A \sim X + P \tag{3}$$

Then, during reversible elec tron tr nsp t

$$A \sim X + BH \quad AH - X + B \tag{4}$$

nd

$$AH - X \quad AH + X \tag{5}$$

Reaction 2 by itself could gi th ADP ATP exchange while reactions 2 and 3 could give the P ATP exchange

It has been thought for a long time that quinoid structures, as exemplified by the flavins, could not only act as ele tron arriers but, because of their structure could also partic pat in phosphate esterification At the present time therefore, a great attempt is being mad by biochemists to identify the X component postulated bove t ascertain whether it comprises one compound or three different compounds, one involved at each site where ATP is formed whether it is som quinoid compound such as vitamin K or even whether it might be identical with the flavins directly involved in electron transport. Briefly this is the present state of the mitochondrial idative problem I believe that the whole mechanism of oxidative phosphorylation will be worked out very soon However at our present state of knowledge, I think it would be more fruitful t discuss a higher order of organization, namely the regulation of the oxidative rate of mitochondria that is brought about by the tight coupling between substrate oxidation, electron transport, and phosphate esterification

This tight coupling was first noted in liver mitochondria by Kielley and Kielley[7] and by Lardy and Wellman These workers noted that fresh mitochondria exh bited very little ATPase activity and very little oxidat ve tivity even when they were presented with substrat Mg^{++} and ATP When some substances that broke down the ATP were dded burst of oxidative activity was noted From the nature of the substances dded it was deduced that the ommon denominat r was th presence of or the production of a phosphate acceptor ADP if this ompound were present, one could obtain ATP formation due to substrat oxidation In ther words, in these fresh mitochondria, ADP formation must occur in order to achiev oxidation of substrate TABLE 1 presents some data on this point. The compound DNP is known to break down ATP by some as-yet unknown mitochondrial mechanisms In fresh mitochondria there is no breakdown of ATP even in the presence of Mg^{++} unless a compound such as DNP is added This being the case, mitochondria, in medium containing ATP nd all the requirements for oxidation, will have very little respiration unless the ATP is broken down to ADP t provide an acceptor for the inorganic phosphate during substrat

TABLE 1

EFFECT OF DNP ON RATE OF GLUTAMATE OXIDATION BY LIVER MITOCHONDRIA

DNP added	Cu mm O_2 uptake/40 min
0	60
3×10^{-4} M	130
1 5 $\times 10^{-4}$ M	350
3×10^{-4} M	460
1 5 $\times 10^{-4}$ M	300

All the flasks contained phosphate buffer cytochrome $MgCl_2$ ATP and glutamat in ppropriat amounts

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oxidation. Not only will added DNP have this effect if hexokinase and glucose are added, glucose-6-phosphate and ADP are formed and respiration increases. If creatine and creatine kinase are added, phosphocreatine and ADP are formed and respiration again is increased By setting up the appropriate conditions, Lardy and Wellman[4] also showed that the concentration of inorganic phosphate could be the limiting condition if this concentration fell below a certain level, even though enough ADP were present, respiration leveled off because no ATP could be formed

The hexokinase and creatine kinase systems are extraneous to the mitochondria the DNP system is, in effect, an artificial one. Potter and I[5] therefore demonstrated that synthetic events in the mitochondria, using ATP could increase the mitochondrial respiratory rate TABLE 2 shows such a result To the medium containing the substrate and ATP we added the other necessary components of the citrulline-synthesizing system, the responsible enzyme being found inside liver mitochondria If is apparent that while this system did not give as high a respiratory increase as did hexokinase a substantial increase was, nevertheless, always observed TABLE 2 also shows the interesting observation that the hexokinase added to the medium could ompete with the citrulline-synthesizing system within the mitochondria for the ATP being formed within the mitochondria.

We also wanted to find out what happened to the ATP that was being broken down by the hexokinase or by DNP during the course of the stimula

TABLE 2

STIMULATION BY CITRULLINE SYNTHESIS OF OXIDATION IN LIVER MITOCHONDRIA

Additions	M citrulline formed	µl O_2 uptake/40 min
None	0	40
Hexokinase, glucose	0	120
Carbamyl glutamate, ornithine, NH_4Cl	3 13	65
Hexokinase, glucose, ornithine, NH_4Cl, carbamyl glutamate	0	100

All the flasks contained phosphate buffer $MgCl_2$ cytochrome c, ATP fumarate, and pyruvat in the appropriat amounts

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TABLE 3
EFFECT OF HEXOKINASE AND OF DNP ON OXYGEN UPTAKE BY LIVER MITOCHONDRIA AND ON NUCLEOTIDE BALANCE IN THE MEDIUM

Additions	μl O_2 30 min	μM ATP in medium	μM AMP and ADP in medium
None	35	2.6	0.4
0.02 ml. hexokinase	45	2.6	0.4
0.04 ml. hexokinase	55	2.6	0.4
0.06 ml hexokinase	73	1.3	1.7
0.08 ml hexokinase	77	0.8	2.2
None	17	2.6	0.2
0.3 × 10^{-4} M DNP	36	2.6	0.2
0.5 × 10^{-4} M DNP	46	2.6	0.2
1.0 × 10^{-4} M DNP	61	2.4	0.4
4.5 × 10^{-4} M DNP	61	0.2	2.4

All the flasks contained phosphate buffer $MgCl_2$ cytochrome c, ATP and glutamate or ketoglutarate in the appropriate amounts

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tion of oxidation by these agents TABLE 3 gives the interesting and somewhat surprising results It can be seen that as the DNP and hexokinase systems are increased, the oxidation of substrate is increased, presumably due to a faster rate of breakdown of ATP However up to a certain level of addition of hexokinase or of DNP the oxidative rate and the phosphorylative rate of the mitochondria can keep pace and thus continuously resynthesize the ATP from ADP and inorganic phosphate as fast as it is being broken down by the added agents I think that these experiments demonstrate within wide limits, the mitochondria can respond to increased loads or demands upon them, whether these demands are external, as in the case of hexokinase or of creatine kinase or internal as in the case of DNP or of the citrulline-synthesizing system It could be said that the oxidative mechanism of mitochondria is responsive to environmental situations and that this response is regulated by means of the tight coupling between oxidation and phosphorylation for it happens that the product of oxidative phosphorylation is also the substrate, so to speak, for synthetic reactions, and the degradation product of these synthetic reactions, ADP is also the substrate, in a sense, for oxidative phosphorylation

There are other methods by which liver mitochondria can be induced to alter their metabolic activity One of the first to be investigated was the effect of altered tonicity Ever since the initial work of Schneider *et al* [10] with liver mitochondria, many other investigators have attacked the problem of altered metabolic activity of muscle mitochondria [11] There is no doubt that the structure of the mitochondria which could be changed easily enough by changing the tonicity of the medium, has a great effect on respiratory and phosphorylative activity However I shall not elaborate on this, because the meanings of many observations are not clear their physiological significance is hazy and in some cases of physiological relevance, such as experiments with thyroxine, the data in the literature are often contradictory However I shall

present some brief and exclusive reports on the chemical meaning of mitochondrial structure.

Isolated rat liver mitochondria, when placed in water, swell up; this swelling is rather distinctive, for these membrane-bound structures do not act like balloons. Water seems to enter the space between the two mitochondrial membranes and, in a short while, the outer membrane begins to detach itself from the inner membrane at a particular spot on the mitochondrial surface. The matrices of the mitochondria become less dense as proteins leak out, and the over-all mitochondrial diameter reaches two or three times normal, due undoubtedly to imbibing water. Something similar to this, although not as drastic, happens even in 0.25 M sucrose solution, particularly if the temperature is elevated. At the same time, the acid-soluble, ultraviolet-absorbing compounds within the mitochondria begin to leak out of them.[illegible] Together with these compounds, which are mostly the adenine nucleotides, AMP, ADP, ATP, DPN, and TPNH,[illegible] the Mg^{++}, Na^{+}, and K^{+} ions initially in the mitochondria come out.[illegible] Also, the ATPase characteristics of the mitochondria are altered from a DNP-sensitivity to a Mg^{++}-sensitivity, and the ability of the mitochondria to couple phosphorylation to oxidation is lost. Some of these phenomena can be prevented and some can be reversed by having the mitochondria do some work, for example, by oxidizing substrate and making ATP,[illegible] and in some cases even by adding Mg^{++} and/or ATP[illegible] or by adding DPN[illegible] to the medium. Even though the functional meaning of these apparent reversibilities is not clear, I wish to emphasize the important part that structure plays in mitochondrial metabolism.

Relevant to the entire discussion of the manufacture of ATP, the following facts are known:[illegible] that liver mitochondria contain ATP; that this ATP is bound, for it can be released only after the mitochondria are damaged; that the phosphates of the ATP are in equilibrium with the phosphates of inorganic phosphate and of ATP-phosphate during oxidative phosphorylation;[illegible] and that, during coupled phosphorylation, the ATP manufactured by the mitochondria is not stored within the mitochondria, for there seems to be present only a certain number of specific binding sites.[illegible] In other words, the liver mitochondria act as secretory bodies for the ATP they manufacture. And, since the sites of the electron-transport enzymes and possibly those of the phosphorylative enzymes are located in, or are a part of, the mitochondrial membranes,[illegible] any alterations of these membranes can have significant effects on the manufacture and secretion of mitochondrial ATP. At the present time, however, one cannot say any more than this, since the experiments relevant to functional regulation of structure have not yet been performed.

In the remainder of this presentation I shall compare liver and heart muscle mitochondria and shall try to show that some of the same factors that regulate the metabolism of liver mitochondria can also be operative in heart muscle mitochondria. An electron micrograph was used to produce FIGURES 1 and 2, the latter showing rat heart muscle fibers. There seems to be no strict localization of the mitochondria either at the level of the A-bands or of the I band. FIGURE 2 shows a typical heart muscle mitochondrion. Heart muscle mitochondria are about the same size and shape as liver mitochondria; they have a



FIGURE 1 Rat myocardium (ventricle) × 11,800

double membrane surrounding them, and also the familiar cristae that in-
vaginations f the inner membrane They also contain small intramitochon-
drial granules similar t those found in liver mitochondria the function of these
gra ules is unknow The only pparent morphological difference is th



FIGURE 2 Rat myocardium (ventricle) × 55,200

the muscle mitochondria have many more cristae than do liver mitochondria, and Palade[14] has pointed out that this might be the morphological counterpart of the increased terminal respiratory activity of muscle over liver mitochondria.

Certain other aspects of the work on heart muscle mitochondria are relevant. Plaut and Plaut[33] found that heart mitochondria could oxidize a variety of substrates of the Krebs cycle however citrate was not oxidized unless DPN was added, and even then a slow rate of oxidation occurred. Despite this finding, these workers had good evidence that the Krebs cycle was operating. Cleland and Slater[34] found that by differential centrifugation they could isolate particles, which they called sarcosomes. These particles, like liver mitochondria, contained a considerable amount of the succinic dehydrogenase and cytochrome oxidase activities of the whole muscle homogenate. Although this fraction contained also a high percentage of the α-ketoglutarate oxidase system, it was found that all of the myokinase or adenylate kinase activity which is found in liver mitochondria was found, not in muscle sarcosomes, but in the sarcoplasm. It could be argued that myokinase, which is easily extracted from liver mitochondria by water, was lost from the muscle sarcosomes during the isolation procedures.

Maley and Plaut[35] showed that heart mitochondria could give as good coupled phosphorylation as does liver mitochondria. These workers demonstrated that the one-step oxidation of α-ketoglutarate to succinate by guinea-pig heart mitochondria gave a P O ratio of 3 4 in the absence of fluoride, which is as good as has been obtained with liver mitochondria. Furthermore the oxidation of glutamate, fumarate, malate and β-hydroxybutyrate gave P O ratios of at least 2 5 whereas succinate oxidation gave 1 8. Holton *et al*[36] obtained similar values from rat heart mitochondria, except that they obtained exceptionally low P O ratios (less than 1) for succinate oxidation. Most workers in this field seem to agree that heart mitochondria do not have an impaired phosphorylative efficiency at least as compared to liver mitochondria. Another means of making this comparison is to test different oxidative phosphorylation inhibitors at low concentrations on the isolated mitochondria of different organs (TABLE 4). In general heart muscle mitochondria are less susceptible to these inhibitors than are the mitochondria from liver kidney brain and skeletal muscle. As the concentration of the inhibitors was increased, both heart and skeletal muscle mitochondria were

TABLE 4
EFFECTS OF VARIOUS INHIBITORS ON P/O RATIOS OF MITOCHONDRIA OF VARIOUS TISSUES

(10^{-4} M)	Liver	Kidney	Heart muscle	Skeletal muscle	Brain
None	3 9	3 6	3 5	3 2	3 3
Dinitrophenol	1 9	2 1	2 2	0 2	1 9
Methylene blue	1 9	2 5	3 3	3 0	2 3
Brilliant cresyl blue	2 0	1 6	2 2	2 3	1 5
Phenosafranine	1 0	1 3	1 9	2 3	2 1
Thionine	0 4	0 8	1 3	2 4	1 5
Janus green	1 0	1 0	1 3	1 8	1 7

All the flasks contained phosphate buffer α-ketoglutarate, AMP Mg^{++} cytochrome c, fluoride, and hexokinase in appropriate amounts.

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TABLE 5
EFFECTS OF ADENINE NUCLEOTIDES AND HEXOKINASE ON THE RESPIRATION OF HEART SARCOSOMES

Adenine nucleotide added	Hexokinase	µl O_2 uptake/15 min
None	−	1 3
None	+	2 4
AMP	−	2 6
AMP	+	5 4
ADP	−	3 9
ADP	+	7 0
ATP	−	3 8

All the flasks contained phosphate buffer fluoride Mg^{++} versene, and glutamate in appropriat amounts
Reproduced by permission of *The Biochemical Journal* [20]

more resistant than the mitochondria from other organs [20] From this aspect isolated muscle mitochondria are hardier than kidney liver and brain mitochondria

Can respiratory control, as exhibited by the acceptor effect and as has been shown by liver mitochondria, also be demonstrated with heart muscle mitochondria? From the data in TABLE 5 it would appear that respiratory control exists in heart sarcosomes, for addition of the adenine nucleotides, with and without the addition of hexokinase, increases the respiratory rate many fold Chance and Connelly[31] and Packer [32] both using ADP found the same thing a two- to threefold increase in respiration, as had Slater and Holton [20] At the same time incidentally Packer[32] found a P O ratio of from 3 t 4 for the oxidation of α-ketoglutarate that agreed with the earlier findings of Maley and Plaut [33] Slater and Holton[20] also found that during the oxidative synthesis of ATP ADP was a much better phosphate acceptor than AMP this finding agreed with the earlier work of Lindberg and Ernster [34] However as I have already mentioned, this result could come about from the possible extraction of myokinase from the muscle mitochondria for in liver mitochondria, both nucleotides act equally as well as phosphate acceptors. TABLE 6 gives some figures bearing more directly on this problem of respiratory control. It can be seen that the classic additions (classic in the case of liver mito-

TABLE 6
RESPIRATORY CONTROL INDEX WITH RAT HEART SARCOSOMES

Additions	µl O_2 uptake/20 min
None	5 0
Hexokinase, glucose	22 5
DNP	23 0
ADP	29 0

All the flasks contained phosphat buffer fluoride, Mg^{++} ATP versene, and glutamat in appropriat amounts
Reproduced by permission of *The Biochemical Journal* [20]

chondria) of ADP DNP and hexokinase-glucose all give in this case a four to sixfold increase in respiration

Another aspect of the same problem involves the breakdown of ATP by fresh mitochondria in the presence of added Mg^{++} Liver mitochondria cannot do this hence if substrate and inorganic phosphate are added no respiration occurs because of the lack of ADP to act as phosphate acceptor Heart mitochondria, as emphasized by Slater and his co-workers, have a higher basal metabolic rat and the reason for this could lie in the observations that added Mg^{++} and added DNP both give equal increases in ATPase activities with heart sarcosomes if both are added together a further doubling of the rate of hydrolysis takes place [■] Of course another reason for the higher oxidative rate could be due to the greater amount of the cytochromes than in the liver mitochondria [■] The stability of the ATPase activities of heart mitochondria parallels those of liver mitochondria, for the DNP effect was not lost after storage for hours at 0° C and the Mg^{++}-activatable ATPase was not decreased after storage for days in the cold [■] However both liver and heart mitochondria will lose their DNP-activatable ATPase if they are kept at elevated temperatures for short periods of time [■] Exactly the same thing happens with both preparations if the α-ketoglutarate oxidase system is tested this system is equally unstable in both preparations For example, rat or t heart sarcosomes will lose this enzymatic activity if kept in sucrose t 25 C for 10 t 15 min [■] On all these bases the quantitative similarity betw en liver and heart muscle mitochondria can be observed readily

The following citations may have some physiological significance Vitale *et al* [■] conducted experiments in which they induced a magnesium deficiency in rats and then measured the phosphorylation efficiency of the mitochondria isolated from heart, kidney and liver They found that only in the case of heart mitochondria was the P O ratio lowered, in this case t about one half that of nondeficient animals They also found that massive doses of thyroxine injected into normal rats produced a lowering of the P O ratio in heart mitochondria, but not in liver mitochondria Both of these effects are reversed by the injection of magnesium Since it is known that continued thyroxine injections decrease the serum level of magnesium, these results indicate (by no means conclusively as the authors themselves acknowledge) the possible existenc of mechanism by which thyroxine can increase th metabolic rate That is, by uncoupling oxidation from phosphorylation through an Mg^{++} mediated effect regulatory device or brake on xidation is lost and runaway respiration could occur

In these days of integrated studies, it is not surprising that studies on the effects of some agent, such as thyroxine on some biochemical functions should be paralleled by experiments to show effects on other parameters in biology in this case, the morphological characteristics of the cell It has been found[■] that thyroxine causes marked swelling *in vitro* as measured by optical density measurements through a suspension of rat liver and kidney mitochondria On the other hand, heart muscle mitochondria are affected only slightly [■] It has been found[■] further that various other agents, such as digitonin, have

In the latter case, phosphocreatine the phosphate donor to ADP must be generated by the action of ATP on creatine thus no net gain of ATP accrues Of course depending on its capacity this system acts as a reservoir of ATP and its release of ATP can be and probably is, physiologically controlled In the long run, however the system can get its supply of ATP only from the mitochondria Therefore it is evident that the mitochondria are the main sources of the ATP necessary for muscle contraction, and that the rate of production of ATP through oxidative phosphorylation can be regulated by the rate of supply of ADP the ADP coming from the actomyosin system of the muscle fibril Muscle working at a low rate supplies a small amount of ADP to the mitochondria which will generate only the small amount of ATP necessary to maintain this low load. If the work of the muscle is increased, more ADP hence more ATP will be supplied enough to maintain the increased work load of the muscle

This limited picture of muscle functioning omits many obvious factors but leads to several conclusions

Any damage to the mitochondria will immediately affect the functioning of the muscle

Any damage to the contractile fibers that produces low output ADP a damage that might be corrected by increasing the input of ATP will not be corrected because the amount of ATP is controlled, in the long run, by the amount of ADP produced by the fibrillar system

It is possible that both the fibrils and the mitochondria become degenerated if they are continuously subjected to a low work load. Muscle atrophy illustrates this, as does the finding, in experiments with liver mitochondria that under identical conditions those mitochondria that have been allowed to function will last longer than those kept from functioning Therefore damage to one component, the mitochondria, will also damage the other component the fibrils, and vice versa. In time this damage may become irreversible, so that even if we could somehow supply the damaged fibrils with undamaged mitochondria to act as ATP donors or if we could supply the damaged mitochondria with new fibrils to act as ADP donors, the fibrils will not contract nor will the mitochondria generate ATP

Despite this pessimistic picture of the failing heart I hope that this deliberately exclusive viewpoint will have shed some light on the biochemical mechanisms involved and that events are not as irrevocable as I have envisaged them

Acknowledgment

I express my appreciation to G E Palade for his loan of the electron micrograph used to obtain FIGURES 1 and 2.

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OXIDATIVE PHOSPHORYLATION STUDIES IN NORMAL AND EXPERIMENTALLY PRODUCED CONGESTIVE HEART FAILURE IN GUINEA PIGS A COMPARISON

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As one tries to formulate a biochemical approach to the heart in failure or to the effect of certain drugs such as digitoxin, one is intrigued by an observation made many years ago with the heart-lung preparation. FIGURE 1 from Evans[1] shows the effect of increasing ventricular pressure on oxygen consumption and the efficiency of output by the heart. At first (curve 1) the diastolic volume and the oxygen consumption (curve 2) show a parallel rise. With increasing intraventricular pressure, however the diastolic volume begins to decline. The interesting fact is that while the diastolic volume is declining the oxygen consumption continues to rise in almost linear fashion. Therefore it appears that beyond a certain ventricular pressure the efficiency of substrate utilization is decreased At higher pressures there seems to be a decrease in the efficiency of transfer of utilizable energy t the muscle. If a heart-lung preparation in this state is treated with digitalis, there is a rise in diastolic volume (curve 3) while oxygen consumption (curve 4) remains about the same From this curve it would appear that, in biochemical terms, digitalis is, recoupling" a system that is "uncoupled

With these considerations in mind we looked for experimental techniques that would make t possible to approach the problem of failure and th effect of certain drugs, such as, digitalis, on a biochemical basis It seemed to us that there would be three general areas where one could look for these effects in the heart first the energy-generating system (oxidative phosphorylation) second, an energy transferring system, such as, myokinase and nucleoside diphosphokinase and, third, the energy utilizing system, for example, actomyosin.

In the initial experiments[2] the yield of oxidativ phosphorylation of cardiac mitochondria from digitalized normal guinea pigs was compared to that of normal untreated animals (TABLE 1)

In these experiments we used three substrates, one of which was α-ketoglutarate with a malonate block this yields a one-step oxidation of α-ketoglutarate t succinate We compared mitochondria from the normal and the digitalized animals and found practically no difference Note also that th oxygen uptake is almost the same in the treated and the untreated animals The same situation prevails with glutamic acid and with acetate as substrates. Thus t would appear that digitalization of the normal guinea pig does not lead to a change in the efficiency of oxidative phosphorylation and has no effect on th rate of substrate oxidation

It seemed possible to us that the effect of digitalis might show p in mitochondria from animals in congestive heart failure. However in order to perform such experiments, it was necessary first t devise a method for pro-

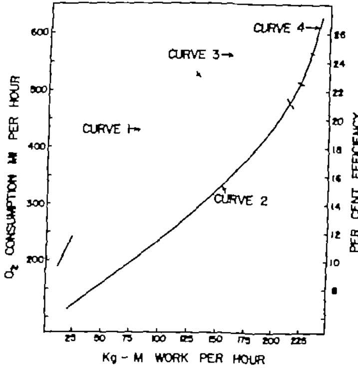


FIGURE 1 Graph showing rise in efficiency (dotted line) when increased output is mediated by enlargement of stroke volume without change in rate or mean arterial pressure Efficiency begins to fail as the limit of diastolic distensibility are approached Continuous line represents oxygen consumption Digitalis added at bar on each curve Reproduced by permission of *The Journal of Physiology* (London) 1918–19 Volume 52

TABLE 1

OXIDATION OF SUBSTRATES AND PHOSPHORYLATION BY CARDIAC MITOCHONDRIA FROM NORMAL AND DIGITALIZED GUINEA PIGS

Substrate	P/O		Q_{O_2}	
	Normal	Digitalized	Normal	Digitalized
α Ketoglutarate + 0 01 M malonate	3 05	3 25	563	539
Glutamate	2 99	2 99	637	665
Acetate	2 32	2 39	320	481

Twenty μmoles potassium acetate plus 1 μmole sodium succinate

ducing failure in animals. Gertler[3] developed such a surgical procedure The manifestations of the congestive heart failure produced in the guinea pig are very similar to those observed in human failure.

TABLE 2 shows a comparison of the yields of oxidative phosphorylation of cardiac mitochondria from normal guinea pigs and from animals with the experimentally produced failure. When glutamate is used as a substrate,

TABLE 2

OXIDATION OF SUBSTRATES AND PHOSPHORYLATION BY CARDIAC MITOCHONDRIA FROM NORMAL ANIMALS AND GUINEA PIGS IN CONGESTIVE FAILURE

Substrate	P/O		Q_{O_2}	
	Control	Operated	Control	Operated
Ketoglutarat + 0 01 M malonate	3 39	1 61(?)	321	136
Glutamat	3 12	3 21	645	615
α-Ketoglutarat	2 70	2 39	555	455

there is no difference in yield of oxidative phosphorylation or in substrate oxidation rate between the normal and the operated animals. The same thing is true with α-ketoglutarate as substrate (in the absence of malonate) In addition, these mitochondria show very little adenosine triphophatase activity when fresh However the adenosine triphophatase activity can be developed up to a maximum level by the addition of graded levels of 2 4-dinitrophenol There is no difference in the degree of development of adenosine triphophatase in response to graded doses of 2 4-dinitrophenol when mitochondria from normal guinea pig hearts are compared to those from animals in experimental failure It would seem from these experiments that there is no difference in the properties of mitochondria from normal and operated animals However we d g t a rather drastic toxic effect of malonate on α-ketoglutarate oxidation with mitochondria from operated animals. In fact, from the verages shown in TABLE 2, one cannot tell that there are many experiments in which there is no oxygen uptake whatsoever with α-ketoglutarate in the presenc of 0 005 M malonate We are interested in this effect of malonate primarily because it is the first difference we have ever found between cardiac mitochondria from normal and operated animals However we have no explanation for the phenomenon at this time, and do not know how it is related to th experimental failure

In general our results are in agreement with those reported by Olson elsewhere in this monograph The process of oxidative phosphorylation (energy generation) does not seem to be affected in congestive failure. What we d not know however is whether in cases of failure in animals there might not be defects n the energy transfer systems that follow the generation of ATP in the oxidative process or in the energy utilizing system (contractile elements) Experiments are in progress to test these systems in the animals in failure

Aside from th possible defects on the enzymatic level within the cell animals in failure, one wonders if there might not be changes in certain physical systems, such as, permeability

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SYNCHRONY OF STRUCTURAL AND FUNCTIONAL CHANGES IN CARDIAC MUSCLE MITOCHONDRIA

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The metabolic factors that influence the contractility of cardiac muscle may be influenced by the osmotic properties of the mitochondria present in the tissue. Heart sarcosomes are the major site of synthesis for high-energy phosphate bonds in heart muscle and these cellular structures have been alluded to as the ionic pump in cells because of the action of energy rich compounds in the control of osmotic properties. Since the work of Raaflaub[1,2] there have been numerous reports[3-6] that adenosine triphosphate (ATP) plus Mg ions can reverse or prevent the swelling of isolated mitochondria. Swelling and shrinking of mitochondria are also known to be accompanied by changes in intramitochondrial water and ions, by changes in light-scattering properties, and by functional changes such as the ability to carry out oxidative phosphorylation.

How does ATP keep mitochondria in the shrunken or contracted state? Since the intramitochondrial regulation of respiratory rate has been shown to be largely under the control of adenosine diphosphate by the work of Lardy and Wellman, Siekewitz and Potter,[7] Chance and Williams,[8,9] as well as as many others, it seemed possible that adenosine diphosphate (ADP) rather *than ATP was responsible for the control of mitochondrial structure.* This hypothesis has been examined by simultaneously monitoring the structural and functional changes in isolated mitochondria with synchronously recording spectrophotometric and polarographic methods. Thus far the results of these experiments have been found to be similar for heart muscle sarcosomes obtained from seven mammalian and two amphibian species. A typical experiment is shown in FIGURE 1. The methods used are as follows. The functional parameters measured are respiration and oxidative phosphorylation as shown, in the trace obtained with the vibrating platinum microelectrode. The oxidation-reduction state of cytochrome *b* shown in one of the spectrophotometric traces was measured by following the specific change due to the oxidation or reduction of this respiratory enzyme at 430 mμ. These changes at 430 mμ are corrected for nonspecific changes in light absorption at a nearby wave length, in this case 410 mμ, using the dual-beam spectrophotometer described by Chance.[8] In the last parameter structural changes were followed by the commonly used practice of measuring turbidity or percentage of light transmission at a nonspecific wave length, 410 mμ in this experiment. These structural changes can be observed at any wave length with a single-beam technique or by measuring scattered light at 90° which has been done in control experiments.

A decrease in optical density here corresponds to an oxidation of cytochrome *b* in the 430–410 mμ trace and to a swelling of the sarcosomes in the 410 mμ trace. The slope of the downward deflection of the platinum microelec

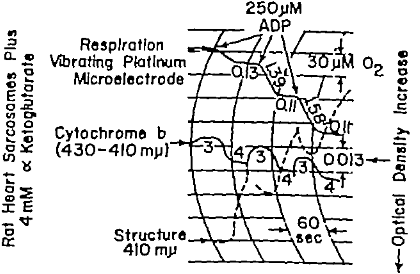


FIGURE 1

trode trace indicates the rate of oxygen utilization The rates of respiration ha been calculated in µmoles O consumed/sec /l and are written below the trac

The preparation used here was composed of rat heart sarcosomes isolated in 0 32 M sucrose media containing 0 001 M Versene at pH 7 4 The air-saturated reaction mixture was of a 3 0-ml volume in 1-sq cm. cuvette t contained 0 05 M sucrose, 0 01 M KCl, 0 02 M phosphate at pH 7 3 and 3 mg sarcosomal protein At the start of th experiment 4 mM α-ketoglutarate was added as the oxidizable substrate and 250 µM ADP was added as the phosphate acceptor The extreme left hand portion of the traces shows the sarcosomes to be in the active state of respiration, called State 3 which is characterized by the presence of phosphate acceptor and substrate this state is accompanied by rapid respiratio and relatively oxidized steady-state of cytochrome b When the added ADP is converted to ATP through oxidative phosphorylation respiration is seen to slow down markedly This change in velocity of electron transport is accompanied by a reduction in cytochrome b and the sarcosomes are now said t be in Stat 4 the quiescent state. This state is characterized by the presence of substrate and by the absence of phosphate acceptor

These aerobic metabolic states of mitochondria are well known from the work of Chance and Williams[4] and constitute no new information here except to point out that the metabolic states described for other mitochondrial systems are equally relevant to heart sarcosomes. What is striking about the State 3 t 4 transition, however is the rapid decrease in turbidity in the single-ended (t 410 mµ only) trace this is due to the swelling of the sarcosomes It is significant to note that the sarcosomes remain in the shrunken or contracted state only as long as the ADP added is being phosphorylated Furthermore

the extent of the light absorption change caused by swelling is very much larger than the specific change observed with cytochrome *b* and the light absorption change would indeed have been very much larger than that shown if it were allowed to continue without interuption

Following this, a second addition of 250 μM ADP was made, causing a State 4 to 3 transition. The following events distinguish this change (1) respiration is increased by a factor tenfold greater than that observed in State 4 (2) this increased rate of electron transport results in an oxidation in the steady-state of cytochrome *b* and (3) a shrinking of the sarcosomes takes place

Mitochondrial shrinking takes place at a rate slower than that of cytochrome *b* oxidation. It can be seen that the sarcosomes did not shrink to their former size this was only because the small amount of ADP added was converted to ATP in approximately 45 seconds that is, a larger addition of ADP would have resulted in the complete reversal of swelling as shown by other experiments. When the intramitochondrial ADP concentration decreases due to phosphorylation, swelling begins again, and the other changes characteristic of the State 3 to 4 transition are clearly seen. A third addition of ADP is accompanied by similar changes

The two complete cycles of ADP addition are accompanied by ADP/O ratios of 3 2 and 3 1 and respiratory stimulations greater than 10 times These ratios are easily calculated from the amount of O_2 utilized during State 3 and the amount of ADP added these results are in agreement with the high phosphorylation efficiency of heart sarcosomes reported earlier by Maley and Plaut and by others Although ATP is being accumulated during the successive periods of phosphorylation shown in the experiment, the rate of swelling of the sarcosomes is unaffected Indeed, the addition of ATP itself appears ineffective in controlling mitochondrial size except by virtue of its ability to form ADP Thus, the addition of Mg ions to sarcosomes incubated in the presence of ATP results in ATPase activity and a consequent reversal of swelling

Other experiments thus far have indicated that reactions that serve to decrease the intramitochondrial ADP concentration result in swelling Thus, oxidizable substrates initiate swelling, and the kinetics of the swelling reactions are found to be related directly to the rate of respiration Conversely reactions that increase the intramitochondrial ADP concentration reverse swelling some examples of these reactions are anaerobiosis, inhibition of electron transport, ATPase activity and exhaustion of oxidizable substrates

In conclusion it has been found that the intramitochondrial ADP concentration is involved in structural and functional changes in heart sarcosomes, as judged by the simultaneous measurement of mitochondrial size respiration, oxidative phosphorylation, and the steady state of the respiratory enzymes, as exemplified by cytochrome *b* These findings indicate that ADP may play a primary role in the control of these processes

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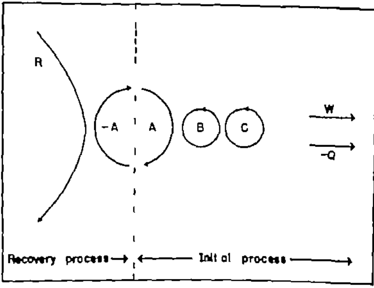


FIGURE 1 Reaction scheme for cycle of muscular activity

The release of chemical energy is reflected in work and heat production. Therefore we turn to the elegant experiments of A V Hill, who sorted out these energy changes. We shall consider muscles of about normal body length and assume that shortening is so small that changes in length-dependent parameters, such as tetanic tension, can be neglected This means that even though the muscle shortens, the length always remains in the region of the broad maximum of the tension-length curve (Ramsey and Street, 1940)

FIGURE 2, from Hill's classic 1938 paper (Hill, 1938) shows how the tension developed by a muscle depends on the speed of contraction The ordinate is the shortening velocity in isotonic contraction, and the abscissa is the load, p or since the velocity v, is constant, it is the force exerted by the muscle. The intercept on the abscissa is the tetanic tension, p_0 The smooth curve is the well-known, hyperbolic force velocity relation,

$$(p + a)(v + b) = b(p_0 + a) \tag{1}$$

where a and b are characteristic constants of the muscle. The rate of doing work, $w = dw/dt$ is the product of the force and the velocity

$$w = pv \tag{2}$$

that is, the product of the abscissa and the ordinate of FIGURE 2. This product vanishes both when there is no load and when the load exceeds the force the muscle can develop, so that the muscle is prevented from shortening. There is an intermediate shortening rate that maximizes the rate of doing work. Clearly the rate at which mechanical energy leaves the muscle is controlled by the speed of shortening.

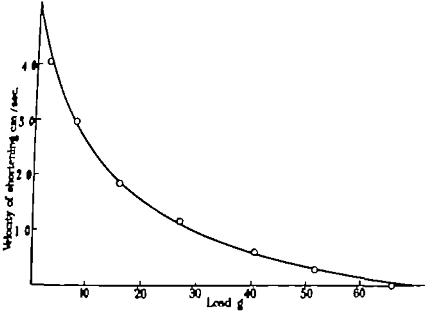


FIGURE 2 Relation between the velocity of shortening, and p the mechanical load in isotonic contraction
Reproduced by permission from *Proceedings of the Royal Society of London* (Hill, 1938)

Energy also leaves the system as heat. FIGURE 3 shows that the speed of shortening also controls this process. In each case total heat output from tetanically stimulated muscle is shown as a function of time before during, and after shortening In the set of experiments in FIGURE 3a the muscle as released 1.2 sec after the start of tetanic stimulation and allowed to shorten various distances under constant load Curve A represents the total heat from an isometric contraction curves B C and D represent the heat for increasing amounts of shortening During shortening the rate of heat production increases relative to the rate for isometric contraction and the total extra heat is proportional to the amount of shortening When the velocity returns to zero (final sections of curves B C and D) the heat flux is essentially the same in the shortened state as in the corresponding isometric state

The heat from the same muscle shortening a constant distance under increasing loads is shown in curves J H G and F respectively in FIGURE 3b The muscle was released at 1 2 sec the end of shortening is indicated by arrows Curve E represents an isometric contraction. Since, at the end of shortening curves J H, G and F are all displaced from the isometric contraction by a constant amount the extra heat associated with shortening does not depend on the velocity of contraction, but *only on the distance shortened*. This means that $-\dot{Q} = -dQ/dt$ the *rate* of heat production, is a linear function of the speed of shortening

$$-\dot{Q} = \phi_0 + av \tag{3}$$

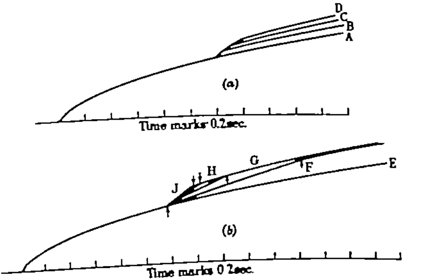


FIGURE 3 Total heat production, $-Q$ as function of time Tetanically stimulated muscle at 0° C See text for the experimental conditions
Reproduced by permission from *Proceedings of the Royal Society of London* (Hill, 1938).

where φ_0 is the rate of heat production in isometric contraction The constant a is dimensionally and numerically the same as the a in the force velocity relation (EQUATION 1) In the following EQUATIONS 1 and 3 will be referred to as the empirical (energy) relations of Hill

The experiments indicated in 3a and 3b can be summarized by saying that heat flux from stimulated muscle depends on the speed of shortening A heat of activation the basal rate for stimulated muscle constrained to remain at constant length

The energy manifested as work and heat derives ultimately from the chemical energy released in reaction A The modulation of the energy flux by the speed of shortening implies that the rate of the driving reaction is also linked to the same parameter To work this out analytically we begin with the first law of thermodynamics,

$$H = \xi\widetilde{\Delta H} = Q - w \tag{4}$$

where ΔH is the change in enthalpy when the extent of reaction A is ξ, $\widetilde{\Delta H}$ is the change in enthalpy for unit extent of reaction, and w is the work done by the system in excess of the expansion work, $P\Delta V$ Differentiating EQUATION 4 with respect to time

$$\dot{\xi}\widetilde{\Delta H} = \dot{Q} - \dot{w} \tag{5}$$

since $\widetilde{\Delta H}$ can generally be treated as a constant quantity The terms on the right hand side of EQUATION 5 can be obtained from the empirical relations of Hill. Combining EQUATIONS 1 2 and 3 with 5

$$\xi = \xi_0 \frac{b + \beta v}{b + v} \tag{6}$$

where $\xi_0 = \phi_0/\widetilde{\Delta H}$ the reaction rate when v (and consequently w) is zero and $\beta = 1 + b(p_0 + a)/\phi_0$ a collection of constant parameters of the muscle. Thus the *chemical* reaction rate ξ is a hyperbolic function of the *mechanical* reaction rate, v

EQUATION 6 can be evaluated numerically by using the empirical observations (Hill, 1939)

$$\phi_0 = ab \tag{7}$$

and

$$p_0 = 4a \tag{8}$$

Then β is equal to 6 and the maximum contraction velocity $v_m = p_0 b/a$ (EQUATION 1) is equal to $4b$ With these relations EQUATION 6 takes the simple form

$$\xi = \xi_0 \frac{1 + 24v/v_m}{1 + 4v/v_m} \tag{9}$$

When $v = v_m$ that is, when the contraction is unloaded the chemical reaction rate, at least in frog sartorius muscle, increases five times relative to the rate for isometric contraction. This relation is plotted as a solid line in FIGURE 4 The abscissa is the relative speed the ordinate, on the left, is the relative reaction rate. The dotted line is $d\xi/dx$ the extent of reaction for a given amount of shortening, and is calculated by dividing the *chemical* reaction rate by the *mechanical* reaction rate. We see that for a given amount of shortening, considerably more chemical energy must be made available if the muscle moves slowly rather than rapidly This means that the driving reaction A is not geared to the displacement of the muscle like a rack and pinion device Rather the mechanochemical coupling is more like a fluid drive that permits slippage between input and output shafts

Kinetics of Model Systems

What chemical systems have these properties? For clues we turn to microscope studies of contracting striated muscle. Photographs made by Huxley and Niedergerke (1954) with an interference microscope illustrate the changing striation pattern as a living muscle contracts isotonically (FIGURE 5) Shortening, from top to bottom takes place almost entirely in the light *I* bands, the width of the dark *A* bands remaining constant This is shown even more dramatically in the experiments of Huxley and Taylor (1955) in which contraction is confined to a single sarcomere stimulated directly with a micropi-

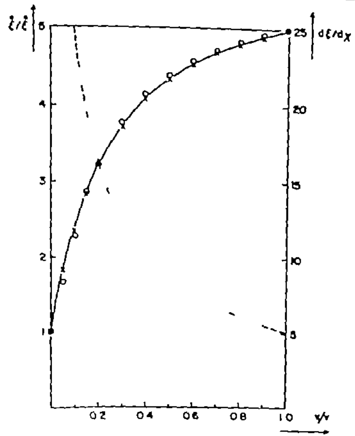


FIGURE 4 Smooth curve relating rate of reaction, ξ/ξ₀ as function of the relative extent of contraction, = /v₀ Corresponding kinetics for sliding model are plotted as circles kinetics for folding model are plotted as crosses (APPENDIX) Both models are scaled to coincide with the smooth curve at = 0 2 and = 1 0 (closed circles)

Dotted curve relation between the extent of reaction for unit distance of contraction, dξ/dx, and the velocity of contraction

pette (FIGURE 6) The picture on the right shows the pipette next to the fiber before stimulation As shown on the left, when current is passed through the pipette, depolarizing the membrane, the *I* band decreases in width until it vanishes, drawing together adjacent *A* bands

Studies at higher magnification with the electron microscope point to a filament d structure of the muscle fibril shown diagrammatically in FIGURE 7 The main point to note is that there are two kinds of filament whose peculiar distribution gives rise to the characteristic striations The *A* band is due to filaments that appear thick in the electron microscope and are thought by some to be made of myosin The second set of filaments, thinner than the first and possibly made of actin, extend from the *Z* line through the *I* band int

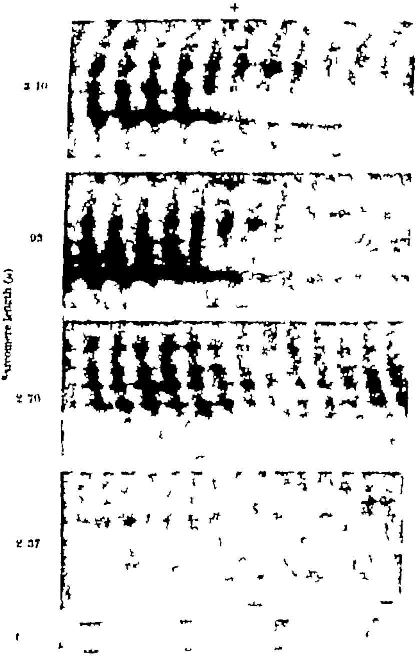


FIGURE 5. Muscle fiber during an isotonic tetanus. A bands are dark; I bands are light. Sarcomere length for each phase of contraction is indicated on the left.
Reproduced by permission from *Nature* (Huxley and Niedergerke, 1954).



FIGURE 6 Shortening of single I band induced by depolarizing small area with micro-pipett (t upper edge of fiber) right, before depolarization left, after depolarization
Reproduced by permission from *Journal of Physiology* (Huxley and Taylor 1955)

the *A* band, there interdigitating with the thick filaments. The thick filaments are definitely discontinuous the thin filaments appear to be continuous, although they are much thinner near the middle of th *A* band, which is probably the reason for the lower inde of refraction of the *H* zone.

Both electron-microscope studies of fixed preparations and light-microscope observation of living muscle suggest that during shortening there is relative motion of thin and thick filament Inspired by these findings, A. F Huxley (1957) noted that if complementary sites were located n the thin and thick filaments respectively and f interaction between these sites depended on their relative position the kinetics of interaction will depend on the speed with which the filaments move past each ther Furthermore, if the interaction between these moving sites were chemically linked with the driving reaction, this could explain how the speed of shortening influences the rat t which energy is supplied for the contraction.

The essential features of this idea are show m FIGURE 8 The thick and thin lines represent the thick and thin set f interdigitating filaments Sites *T* are on the thick filaments and sites *t* are on the thin filament The *T* and *t* sites are complementary that is, some chemical interaction can occur between them The interaction probability per unit time *H*( ) is assumed to depend on the relative position of the sites This is schematized by the dotted, bell shaped curve Suppose the thin filament moves past the thick one then

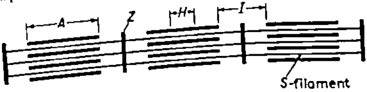


FIGURE 7 Diagram of muscle fibril
Reproduced by permission from *Progress in Biophysics* (Huxley 1957)

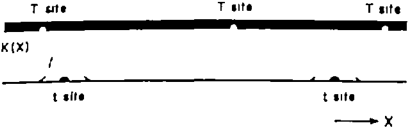


FIGURE 8. Thick and thin filaments of muscle fibril. See text for description.

as a t site approaches a T site the probability of interaction increases until the sites are directly opposite each other. As the motion continues, the interaction probability decreases, finally reaching zero when the distance exceeds the radius of the bell. For a given amount of shortening, there will be more interaction the slower the relative velocity since the extent of interaction is proportional to the time available for interaction. As shown in FIGURE 4 this is what happens, qualitatively at least, in living muscle.

We do not know the nature of the T-t interaction. Fortunately this does not block further exploration of the model because, in order to push ahead, we need to know only the statistical nature of the interaction. We shall assume that in the course of a transit of a t site past a T site the interaction event (1) either does nor does not happen, and (2) can happen only once. For example, suppose t is a binding site for adenosine triphosphate (ATP) and T is an ATPase site. Then, as the sites move past each other the ATP either does or does not get split. The probability for interaction (splitting of ATP) is greater the slower the speed, but the reaction can happen only once for each molecule. If this reaction is the rate-limiting step in the chain of coupled reactions comprising the initial process (FIGURE 1) it can control the rate of the reaction sequence. In this case the T-t interaction rate can be equated to $d\xi/dt$.

To derive the kinetics of the T-t interaction process, consider a pair of sites moving past each other with relative velocity u. Suppose the displacement of t relative to T is x. Then the probability that interaction will have occurred already is n. The probability δn that t and T will interact in the next element of time δt is the product of $(1 - n)$ the probability that they have not yet interacted and $k_x\delta t$ the a priori probability factor for interaction or

$$\delta n = (1 - n)k\,\delta t \tag{10}$$

Since δx the distance traversed in the element of time δt is $u\delta t$

$$\delta n = (1 - n)k_x\,\delta x/u \tag{11}$$

Integration across the entire transit gives n the probability for interaction in a complete transit at velocity u

$$n = 1 - e^{-\lambda/u} \tag{12}$$

where $\lambda = \int_{-\infty}^{\infty} k\,dx$ the area under the bell-shaped probability curve. The

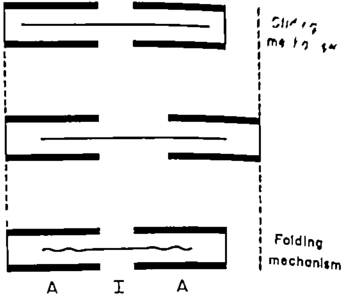


FIGURE 9 Sliding and folding contraction mechanisms center sarcomere before contraction top and bottom, sarcomere after contraction

result is intuitively satisfying when the speed s is infinite, the exponential is unity and the probability for interaction, π vanishes when s is zero the exponential vanishes and π is unity We also notice that the particular shape of the probability curve does not matter In principle the curve could have had an asymmetrical shape instead of the symmetrical one chosen for illustration

Before this analysis can be applied to contracting muscle the distribution of sites and of relative velocity along the two kinds of filaments must be decided. We shall assume that the sites are distributed with uniform linear density along each set of filaments Each T-A interaction is assumed to be independent of previous interactions

The relative velocity of sites along the two sets of filaments will depend on the relative motion of the filaments during contraction Current models for contraction can generally be classed as either sliding or folding models (FIGURE 9) In both models contraction is accompanied by shortening of the I band, the A band width remaining constant, in agreement with the microscopical evidence In the sliding model the contraction force arises from interaction between thick and thin filaments The relative velocity is the same along the entire region of overlap In the folding model the ends of the thin filaments are assumed to be fixed relative to the thick filaments during the contraction. The force in this case, stems from a change in the elasticity of the thin filaments As opposed to the sliding model however the relative velocity of A and T sites now depends on their position along the filament At the free end of the thin filament the relative velocity is zero If the folding acts as in a mechanical spring, the relative velocity increases linearly along the filament, reaching a maximum near the I band.

Do these differences in the contraction mechanism lead to corresponding differences in the dependence of the interaction rate of the whole muscle on the speed of shortening? The answer depends on how much we know about the interaction sites If we try to fit the predictions of the sliding and folding mechanism to the thermodynamic consequences of Hill's energy relations, we can do so remarkably well with both models (FIGURE 4) The circles show the dependence of the *T-t* interaction rate on the muscle contraction rate using a sliding model the crosses were calculated from a folding model (APPENDIX). These can be compared with the solid curve derived from experiment The solid points were used for fitting We see that both models can be adjusted to fall well within experimental uncertainty although if a choice must be made on mathematical grounds, the folding model provides a slightly better approximation to the hyperbola than does the sliding model

To make the calculations agree with the hyperbola at relative velocities of 0.2 and 1 0 different values must be assumed for the number of *T* sites per unit volume and the distance between *t* sites (APPENDIX) However since we have no independent values for these quantities, this cannot be used to differentiate between the two contraction mechanisms We must conclude that the chemical kinetics derived from Hill s energy relations can be accommodated by *both* models with equal facility

The Contractile Mechanism

We now consider the interaction between the moving filaments on the molecular level In the sliding model the interaction between filaments generates the contractile force (Huxley 195) and also regulates the flux of chemical energy The site on the thick filament is placed on an oscillating "side piece which can attach itself to a complementary site on the thin filament If the attachment is made when the oscillating site is to the right of its equilibrium position, it will tend to pull the thin filament to the left Conversely if the connection occurs when the side piece is on the other side of its equilibrium position, it will tend to pull the thin filament in the opposite direction T get unidirectional (shortening) tension it must be assumed that the probability of attachment and detachment is asymmetrical with respect to the equilibrium position of the oscillating site Huxley showed that if the appropriate ymmetrical interaction probability functions were postulated, the tension ould depend on the speed of shortening in the way described by Hill s force velocity relation (EQUATION 1)

The folding model however requires no such restriction Assume, for example, that the contractile force in the thin filament stems from n unbalance in the electrostatic and entropic forces in a flexible polymer Riseman and Kirkwood (1948) and Morales and Botts (1952) made this assumption in discussing related models The argument presented begins with the uncharged filament (FIGURE 10a) The ends of the filament will tend toward the position that maximizes the number of configurations the filament can assume This tendency the entropic force will draw the ends of the filament together How

Neither model, however ccounts for the heat of ctivation, ...

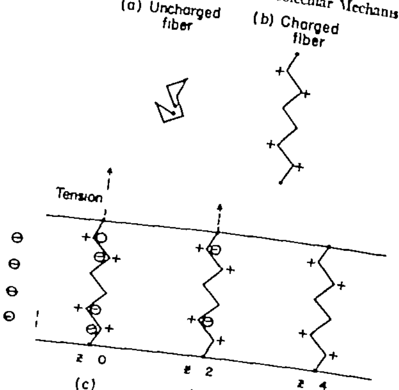


FIGURE 10. The electrostatic-entropic mechanism for generating tension. ⊖ particles carrying unit negative charge. (a) uncharged filament folded by entropic force. (b) positively charged filament at equilibrium length—the entropic force is balanced by electrostatic force. (c), (d) and (e) negatively charged particles bound to positively charged filament. Net remaining charge is . . . Tension developed by filament is proportional to extent of charge neutralization.

ever, if charges are distributed along the fiber, the entropic force will be opposed by an electrostatic force tending to maximize the distance between charges. The equilibrium length strikes a balance between the entropic force tending to shorten the filament and the electrostatic force tending to stretch it (FIGURE 10b). If depolarization of the muscle membrane makes available negative charges that can stick to the filament, the filament will tend to shorten since the electrostatic force will no longer balance the entropic force (FIGURE 10c).

Suppose that, as the filament shortens, the attached charge can be detached by interaction with passing sites. For example, assume that the negative charges are carried by ATP molecules that stick to the t sites on the thin filaments. As above, the interaction with the T sites on the thick filament (not shown in FIGURE 10) can be hydrolysis followed by desorption of the products. Then, after each interaction there will be a finite time until the

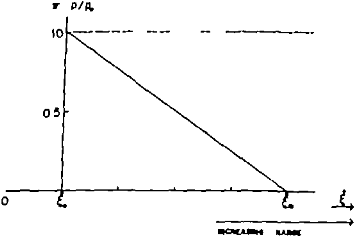


FIGURE 11 The relative tension, p/p_0 as function of the rate of *T-I* interaction the latter has been equated to ξ, the rate of chemical reaction p_0 = isometric tension, ξ_0 = rate of chemical reaction when the contraction velocity is zero, ξ_m = rate of chemical reaction when the contraction velocity is maximum

next ATP sticks on, when the *I* site is vacant In this way the average number of ATP molecules on the thin filament at any time will decrease as the interaction rate increases This simple mechanism relates tension to the interaction rate since, in the first approximation, the tension developed by the filament will be proportional to the amount of neutralization of positive charge by negative charge adsorbed on the *I* sites (FIGURE 10*c d* and *e*) Thus the relation between relative tension, p/p_0 and reaction rate, ξ shown in FIGURE 11 *follows quite naturally from the electrostatic-entropic mechanism* of tension development. The tension will be maximum when the chemical reaction rate is minimum, for then the positive charge on the filament will suffer the greatest amount of charge neutralization by adsorbed negative charge. *As the reaction rate increases, decreasing the average amount of* neutralization the tension decreases Stated algebraically

$$\xi - \xi_0 = \frac{\xi_m - \xi_0}{p_0}(p_0 - p) \qquad (13)$$

where ξ_0 and ξ_m are the reaction rates for isometrical and unloaded contractions, respectively

The model is now complete and can explain quantitatively the velocity dependence of the mechanical and thermal energy fluxes in intact living muscle The argument rests on the assumption that the limiting factor in ξ, the rate at which chemical energy is made available to the contracting tissue is the rate of reaction of substances constrained to sites in relative motion, that is, the rate of *T-I* interaction Since the kinetics for interactions of this type were shown to be homeomorphic with a hyperbolic relation be-

The abscissa of FIGURE 11 is scaled so that ξ_m/ξ_0 = 5 as in frog sartorius muscle

tween ξ and the speed of shortening, v (FIGURE 4 and APPENDIX) the function $\xi(v)$ can be approximated by EQUATION 6. The function $\xi(p)$ is taken from the relation between chemical reaction rate and tension, p, inherent in the electrostatic-entropic mechanism for developing the contractile force (EQUATION 13). The characteristics of the muscle can then be obtained as follows. The classic force-velocity relation (EQUATION 1) results from elimination of ξ between EQUATIONS 6 and 13 and the identification

$$a = p_0 \frac{\beta - \xi_m/\xi_0}{\xi_m/\xi_0 - 1} \tag{14}$$

Using the force-velocity relation we can calculate w, the rate of doing work, as a function of the speed of shortening. Then the heat production, $\dot{Q}$, can be derived from the first law of thermodynamics (EQUATION 5) and the kinetics of the T-t interaction process (EQUATION 6).

I should like to emphasize the fact that my analysis is not wedded to the electrostatic-entropic mechanism for development of the contractile force. It was chosen simply to illustrate a *class of mechanisms* in which the contractile force varies linearly with the amount of some molecule bound to the thin filament, and in which the bound molecules can be "brushed off" in passing sites on the thick filaments.

The remarkable thing about this kind of mechanism is that the characteristics of the living tissue are derived from quite general considerations. The correct equations flow from the structure rather than the details of the model. There are, of course, a number of disturbing loose ends that need tying, but I feel that such an accommodating model deserves to be in the thick of the competition.

APPENDIX

THE DEPENDENCE OF THE T-t INTERACTION RATE ON THE SHORTENING VELOCITY FOR SLIDING AND FOLDING MODELS OF CONTRACTION

Notation

l = distance between t sites on the thin filament

M = number of T sites/cc. of muscle

$\dot{r}$ = T-t interaction rate/cc. of muscle

In ATP induced contractions of muscles extracted with glycerol, the H zone appears to decrease in size, suggesting that in this preparation the ends of the thin filaments are not fixed with respect to the thick filaments and the contraction is effected by a sliding mechanism (Huxley and Hanson, 1954). This implies that the size of the H zone should be proportional to the length of the I band in myofibrils of different sarcomere lengths. However, in myofibrils from frog muscle, the H zone is apparent in sarcomeres of a wide range in lengths, and there is no simple correlation between the lengths of the I band and the H zone (Carlsen and Perry, 1958).

A second point of controversy is whether both sets (sliding model) or a single set (folding model) of filaments are necessary for tension development. In this connection, the fate of the ATP induced contraction upon differential extraction of the muscle, removing only the thick filaments, is of particular interest. The experimental results are not clear. In some studies the ATP response is lost when the thick filaments are extracted (Huxley and Hanson, 1954), while in others there is limited contraction upon addition of ATP even when substantially all of the ATPase is removed (Carlsen and Perry, 1958). The latter, of course, would not be unexpected if tension were developed by the electrostatic-entropic mechanism developed above. Clearly these points require further experimental study.

n = probability of T-t interaction in a complete transit
= ratio of the distance of t site from the fixed end to the length of the folding region (in the folding model)
= length of sarcomere
v = velocity of t sites relative to T sites
= velocity of contraction of the ends of the muscle (expressed in muscle lengths/sec)
λ = area under the probability curve for prior T t interaction

Sliding model. In the sliding model the velocity, of t sites on the thin filament relative to T sites on the thick filament is the same along the length of the filaments. This velocity is related to the rate of shortening of the intact muscle, by the expression

$$= s/2 \tag{15}$$

here is the sarcomere length. If the distance between t sites is l each T site will be presented with $/l \times t$ sites/unit time. Therefore the interaction rate/cc of muscle is $M n s/l$ where M is the number of T sites/cc of muscle, and is the probability of T-t interaction in transit to relative velocity. Using the function ( ) from EQUATION 12, the number of T t interactions/cc of muscle/unit time is

$$N = \frac{s M}{l}(1 - \quad ^{-\lambda/s}) \tag{16}$$

Combination of EQUATION 15 with 16 gives the rate of chemical reaction as function of the speed of shortening of the ends of the muscle.

Folding model. In the folding model the velocity of t sites relative to the T sites will be zero at the fixed end of the thin filaments and will be at the end of the folding region closest to the I band, where, as in the sliding model, the shortening velocity of the sarcomere is 2. Therefore, if is the ratio of the distance of given t site from the fixed end to the length of the folding region, its relative velocity will be rs. A T site at the corresponding part of the thick filament will suffer rs/l transits, where l is the distance between t sites. Therefore the reaction rate/cc of muscle is

$$N = \frac{M}{l}\int_0 r(1 - \quad ^{-\lambda/rs})\,dr \tag{17}$$

Carrying out the integration,

$$N = \frac{M}{2l}\left(1 - \quad /^{s} + {}^{\lambda}\left[\quad /^{s} + {}^{\lambda} E_i\left(- {}^{\lambda}\right)\right]\right) \tag{18}$$

where $E_i\left(-\frac{\lambda}{}\right)$ is the exponential integral $\int^{\lambda/} \left(\frac{-}{}\right) ds$. The function $V(\ )$ result from combination of EQUATIONS 15 with 18.

Fitting the sliding and folding models to the hyperbolic kinetics derived from Hill's empirical relations. It has been shown that the rate of the driving reaction for contraction is hyperbolic function of the shortening speed (EQUATION 9). To compare this function with the kinetics of the sliding and folding models, values were chosen for the quantities M/l and λ to make EQUATIONS 16 and 18 agree with the hyperbola at $/r = 0.2$ and $/r = 1.0$. Different values of M/l and λ were used for each model. $(M/l)_{\text{sliding}}/(M/l)_{\text{folding}} = 0.45$; $\lambda_{\text{sliding}}/\lambda_{\text{folding}} = 2.0$. Having selected M/l and λ, values of V were calculated according to EQUATIONS 16 and 18 and plotted as circles and crosses, respectively, in FIGURE 4.

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MUSCLE PROTEINS AND ENERGY UTILIZATION

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I wonder if the difficulty that one faces in trying to correlate the structure and enzymatic activity of muscle proteins with muscle function and with the utilization of energy in muscle is sufficiently realized. Ordinarily the enzymologist has a fairly straightforward task. He begins with the knowledge that a certain organ or tissue performs a certain biochemical function; he then proceeds to prepare slices, breis, and extracts from the tissue and in isolating a system he is guided by the chemical reaction he has in mind.

However, the muscle or heart biochemist or physiologist is in a much more difficult position, as he is interested in the entire physiological or biophysical process of contraction. He soon encounters a form of the complementarity principle. This principle is a fundamental one of modern physics, and it sets certain limits to our inquiries and to our knowledge. As stated by Niels Bohr: "Evidence obtained under different experimental conditions cannot be comprehended within a single picture, but must be regarded as complementary in the sense that only the totality of the phenomena exhausts the possible information about the objects." Thus the behavior of light can be described, depending on the experimental arrangements, either as a wave or as a stream of particles. As another example, it is impossible to measure *at the same time* with very high accuracy both the position and the velocity of an electron. Either, however, can be determined separately. This sort of complementarity may be operating when one studies muscle. One may obtain the type of information that was produced by the brilliant work of Hill and his school[2] who approached the muscle with the tools of the classical physiologist, including the most intricate measurements of thermal, electrical and mechanical phenomena. If, however, a biochemist approaches the muscle, he will first tear it apart and, with luck, get some biochemical information; however, it will be extremely difficult to say anything about the place of the chemical phenomena in the context of contraction. In the past, by the use of these two approaches, much detailed information has been accumulated by the physiologists on the one hand and by the biochemists on the other. However, it seems to me that today's most urgent task is to attempt to achieve a synthesis between muscle physiology, ultrastructure, and biochemistry. We can only hope that this is possible, and we can at least pursue our inquiries to the limit set by a principle similar to that of complementarity.

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This is not an exhaustive review of the subject, but merely a discussion of some problems of muscle biochemistry that are of particular interest to the author in his own research, or that appear to be particularly difficult to solve. The reader wishing to gain a fuller orientation in this field is referred to any one of several recent excellent reviews.

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It should be noted that the principle of complementarity in its proper that is quantum mechanical, sense may also be plaguing us. The concepts of classical chemistry and physics may soon be inadequate in describing an intricate system such as muscle and we shall have to turn to the proper use of quantum mechanics if we hope to achieve a valid description. We owe a tremendous debt to Albert Szent Gyorgyi who has been a champion of this idea for the last twenty years

In any attempt to bridge the gap between physiology and biochemistry in the case of muscle it is apparent that the first problem lies in deciding (1) which proteins and enzyme systems that can be obtained from a muscle extract have a rol in contraction proper (2) which are possible artifacts and (3) which function merely as nonspecific suppliers of energy in the form of high-energy phosphat ompounds I think the sorting out of those proteins and enzymes that fall in the third class is relatively simple sinc we know much about the processes responsible for the generation of energy both in the presence and in the absence of xygen these processes receive considerable attention elsewhere in this monograph Therefore hen we speak about proteins oncerned with muscle contraction we think in terms of those prot ins that om into their own fter the proteins that supply energy have done their job

Recent developments muscle research The last few years have brought a tremendous advance in this field this is due t several factors First, there was the discovery of certain muscle models, that is systems that stand halfway between the whole muscle that was the object of classical physiology and the protein and enzyme preparation studied in th biochemical laboratory As a result of Albert Szent-Gyorgyi s discovery it is kno n that one can extract a muscle with 50 per cent glycerol nd store it for n almost indefinit period of time n a deep freeze, and still obtain ontraction When this fiber having been removed from the freezer and washed out with appropriate salt solutions, is brought together with adenosine triphosphate (ATP) one obtains a good contraction The analysis of this contraction has been carried out by various groups Weber and his school in Germany have done a particularly thorough job in showing that many of the properties of the contraction of the glycerinated fiber are identical w th those observed in living muscle One phenomenon that is particularly onspicuous is the so-called phenomenon of quick release If a stimulated muscle is suddenly released to shorter length, the tension will drop considerably but after this sudden drop there will be a slow rise to a new equilibrium level corresponding to the new length. The same phenomenon can be observed on a glycerinated muscle fiber There are other similarities, and t is reasonable to say that the glycerinated muscle fiber can be considered fairly good link between the whole muscle and the soluble systems

Many ad ances in our knowledge of the muscle protein ha also occurred New muscle proteins have been discovered, and properti of those known for some time ha been clarified It is self-evident that before one mak omparisons between whole muscle nd the proteins extracted from it, one must know much more about these proteins, their structure, and their func

tion. The third area in which great advances have been made is that of the fine structure of muscle. The important features of this work are the attempts to determine the localization in the muscle fiber of proteins obtained *in vitro*.

The fourth area in which interesting developments have occurred in the last few years is in the study of chemical changes in muscle during contraction. Work of this type has been done in various laboratories, including those of W. F. H. Mommaerts, University of California Medical School, Los Angeles, Calif.; Carlson's; and my own in collaboration with A. Caspó of The Rockefeller Institute, New York, N. Y. These studies have raised interesting and perplexing questions, and nature, so far, has refused to give a direct answer to the question: "What chemical changes occur in a single twitch?"*

I now return to the simpler things, which actually turn out to be not so simple; that is, to the proteins that can be studied in solution. The term myosin has been in use for nearly one hundred years, having been introduced in 1859 by Kühne,[8] although its original meaning was different from the sense in which it later came to be used. Until the discoveries of Albert Szent-Györgyi and Straub[9a] in the early forties, myosin denoted the substance or substances that could be extracted from ground muscle at a fairly high ionic strength, about 0.5 M, but were insoluble at low ionic strength. In 1930 von Muralt and Edsall[9] showed that such an extract possesses a very strong double refraction of flow, indicating the presence of elongated, rather large, molecules. In the meantime, the lactic acid theory of muscle contraction had been discarded and the phosphate-containing compounds that today are commonplace to biochemists came to the forefront of interest. During the late thirties and early forties several groups were able to show that "myosin," the viscous extract obtained from muscle residue, shows some remarkable reactions with ATP. It was discovered that, on the one hand, ATP has a profound effect on the physicochemical properties of myosin. The double refraction of flow disappears, or considerably decreases, on addition of ATP and threads prepared by squirting myosin into water contracted with ATP. On the other hand, ATP itself lost a phosphate group when it was brought together with this muscle extract. These discoveries are associated with Needham *et al.*,[10] Engelhardt and Ljubimova,[11] and Szent-Györgyi and his co-workers.

Thus it was apparent that ATP can affect the mechanical and physicochemical properties of the muscle proteins, and that the proteins, or the protein as it was thought in those days, can in turn act enzymatically on ATP. The tempting thought arose that these must surely be the reactions that underlie muscle contraction. The work of Lundsgaard had already shown that muscle contraction is somehow linked to the breakdown of organic phosphate compounds.

Myosin-B and "reconstituted actomyosin." It was soon found that the substance re-named myosin-B by Szent-Györgyi and his colleagues[2] is not the only interesting substance obtainable from the muscle residue. It was possible

* An important discovery bearing on this problem was made by Davies and his colleagues[8] since the preparation of this paper.

to obtain a protein that had the ability to split ATP but the protein showed no change under the influence of ATP. This new protein became known by several names: myosin A, L myosin, crystalline myosin, or simply myosin. Still another protein, actin, can be extracted from muscle and it can react with myosin to give a product that is very similar to the old myosin-B. Actin itself exists in two forms, a globular form and a fibrous form. Under the influence of salt the globular is transformed into the fibrous form. It is the latter that interacts with myosin and forms what may be called reconstituted actomyosin.

Myosin-B, the old myosin, has been considered in general to be actomyosin. Indeed it behaves very much like a combination of actin and myosin although until recently there has not been much direct proof that it actually consists of myosin and actin. Some work offers good circumstantial evidence[n, n] in favor of this view but it seems important to settle this question definitely. One studies the combination of myosin and actin because one believes that this complex is the same as myosin-B that one extracts from muscle and that is believed to be the contractile protein. One must be sure that there is no gap in this chain. A. Weber[n] has conducted some experiments that, to my mind, make this position strong. Recently however owing to the work of von Hippel *et al.* and of Kominz *et al.* the problem of the identity of actomyosin and myosin-B has become rather crucial. The following are experimental data obtained in my laboratory by A. Martonosi.

It appeared that the simplest approach would be to attempt the isolation from myosin-B of a substance having the properties of actin. Martonosi succeeded in doing this. He prepared myosin-B, treated it with acetone, and then extracted this dry acetone powder with distilled water in the same way as one does in the preparation of G actin except that in the latter extraction, one treats the muscle residue itself with acetone. He obtained a clear solution of low viscosity that, on addition of salt, showed an increase in viscosity: this has been known to be a property of G actin. Furthermore, this substance when added to myosin, increased the viscosity and the intensity of the scattered light; both changes were reversed by ATP. Moreover the protein isolated from myosin-B strongly increased the ATPase activity of myosin at low ionic strength in the presence of Mg^{++} (TABLE 1). Therefore in all these respects the isolated compound behaved very much like genuine actin.

We then turned to one of the most specific methods in biology: immunology. Immunochemical methods have been brilliantly put to use by Holtzer and Marshall[n] in another area of muscle research. This method proved quite useful in our work. We prepared an antibody against F-actin by injecting the rabbit protein into a rooster and obtained a serum which, at a high salt concentration, gave a good precipitate with actin. There was no cross reaction with myosin and tropomyosin; therefore we felt fairly confident that this antiactin was specific. When the antiactin was brought together with the protein isolated from myosin-B a precipitate formed, confirming the results just mentioned (TABLE 2). Finally, in corroboration, the amino acid com-

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T BLE 3

PRECIPIT TE FORMATION ITH CHICKEN ANTI ACTIN

Antigen	Reaction
Myosin	−
F-actin (Straub-Mommaerts)	+
F-actin from myosin-B	+
Tropomyosin	−

Incubation of rablat antigen th antuserum t 38° C for 60 min followed by standing at 2° C for 18 hours The incubation mixture contained 1 8 M KCl and 0 01 M tris(hydroxymethyl)aminomethane buffer pH 7 4 The antiserum was diluted 1 1 and the antigen concentration ranged from 0 03 mg /ml to 0 25 mg /ml

two kinds of components, only one of which is the carrier of ATPase [81-84] Andrew Szent-Gyorgyi[84] has suggested the term 'meromyosin for the product obtained with trypsin digestion, but in view of the fact that now it is possible to obtain similar but not quite identical fragments in many ther ways, such as chymotrypsin[85] and subtilisin digestion,[86] the term may have outlived its usefulness From every point of view the fraction that has ATPase ctivity seems to be the business end of myosin, because it is not only an ATPase, but because it is also able to react with actin this has been demonstrated in th ultracentrifuge by Mihalyi and Szent-Gyorgyi [88] Molecular weights f these fragments obtained by proteolytic digestion are still not settled definitely but I am inlined to accept the most recent measurements btained by various methods in my laboratory [87] which are confirmed by Susan Lowey on th B logy Laboratory of Harvard University Cambridge, Mass and t say that the most likely values are about 320,000 fo the heavy and about 120,000 for the light fraction, so that one of each of th two kinds of fragments ppears on digestion of one myosin molecule It seems that there must exist two fairly distinct regions in the myosin molecule and as a result of th ction f proteolytic enzymes, these regions become separated and partly degraded Th intact parts—the greater part of them is undo btedly left intact—appear as what loosely have been called meromyosins

There has been some question as t whether the proteolytic enzymes that produce various meromyosins indeed act as proteolytic enzymes Middlebrook [89] I think, is inclined to believe that they produce their effect by some unknown mechanism, but I should say on the basis of our own work[87] and that of Gladner and Folk [90] that the C terminal groups appearing after trypsin and chymotrypsin treatment are the very ones that could be expected if the enzymes had acted as specific proteolytic enzymes

Actin Actin can be obtained from a muscle residu from which myosin and all th ther soluble proteins have been extracted. After this residue has been treated with mild alkali and acetone, the dry residue can be ex tra ted with water this aqueous extract contains the globular form of actin. As I mentioned earlier salts change the G-form int another form that behaves in many respects as substances of high molecular weight are known to behave, and has been termed F-actin (F for fibrous) It appears th[illegible]

is in some way involved in this polymerization reaction, and it may be an integral part of the actin molecule The presence of ATP in actin was first demonstrated by Straub and Feuer and subsequently by Laki *et al* [30] and by Mommaerts.[31] It appears that globular actin contains approximately one mole of ATP for each mole of G-actin, having a molecular weight of about 60,000 on polymerization ATP is transformed into adenosine diphosphate (ADP) [32] It seems that *in vitro* ATP is essential for maintaining the G-actin in a polymerizable form, but the F-actin is much more stable.

Reaction of actomyosin with ATP and pyrophosphate. One of the most striking properties of F-actin is its ability to form a complex with myosin This so-called actomyosin has a much higher viscosity than would correspond to the sum of the viscosities of the two components and has a marked double refraction of flow Its ATPase activity in the presence of Mg^{++} far exceed that of myosin.

As mentioned above in the early phases of the more recent investigations on muscle proteins several research groups found that myosin-B which we would now call natural actomyosin, undergoes striking physicochemical changes in that its double refraction of flow disappears and its viscosity drops markedly when ATP is added Similar changes have been found to occur in light-scattering studies [33, 34] During the last few years it has become an important problem to decide what actually happens when ATP is added to either the natural or the reconstituted actomyosin [35] The significance of the problem lies in the fact that it is conceivable that the interaction between actin myosin, or actomyosin, and ATP and the various ions may give us a clue to the events that take place in muscle if these proteins are indeed those responsible for muscle contraction. The phenomena occurring in the actomyosin ATP system depend very strongly on the ionic strength of the medium At low ionic strength actomyosin is hardly soluble and, under these conditions, one can study either suspensions or threads Addition of ATP will produce a synaeresis of the suspension or a contraction of the thread In fact, the observation originally made by Albert Szent-Gyorgyi[7] of ATP-induced actomyosin thread contraction is one of the most suggestive pieces of evidence favoring the view that actomyosin is involved in contraction in the whole muscle

However at a higher ionic strength, about one-half molar for example it is possible to study the previously mentioned changes in the viscosity light scattering, double refraction of flow It is, of course, of great interest to know what molecular changes underlie the bulk changes It has been assumed for a long time that ATP and pyrophosphate and some other substances cause the dissociation of actomyosin into actin and myosin My own opinion is that, in fact, this is the case my view is based on light-scattering experiments that show a decrease in the average molecular weight on addition of ATP [34, 35] and also on the recent work of Martonosi in our laboratory By using a differential ammonium sulfate salting-out technique he was able to show that whereas an actomyosin solution shows one peak at about 30 per cent saturation in the presence of pyrophosphate two such peaks are obtained and their positions very closely correspond to the position of the peaks of myosin and actin (FIGURE 1) If it is indeed the case that ATP or pyrophosphate causes

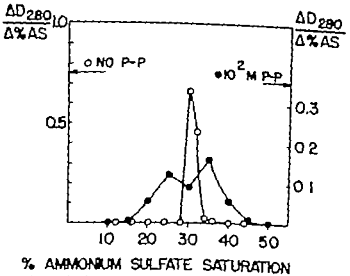


FIGURE 1 Differential salting-out curves of myosin B and myosin B + pyrophosphate Equilibration with ammonium sulfate (AS) at 0° C for 24 hours the precipitate was centrifuged at 20,000 g for 30 min and the optical density (O D) of the supernatant was measured in Beckman DU spectrophotometer at 280 mμ

the dissociation of the actomyosin complex and if this is essentially what happens even at the ionic strength prevailing in muscle the in vitro studies may be of great significance in understanding some of the proposed muscle models and would provide a molecular mechanism of muscular contraction

Tropomyosin Tropomyosin is another protein assumed to be in some way involved in contraction Its role is rather uncertain although, on the basis of its amino acid composition Bailey[26] originally suggested as the name implies, that it might be a building block of myosin This idea has recently been elaborated upon with considerable imagination and much hard work by Laki[27] He suggests that tropomyosin and actin, together with some other not completely characterized proteins, are the real subunits of myosin

Heart myosin I shall now add something about heart muscle proteins. Fundamentally the heart actomyosin system seems to behave very much like the skeletal one Myosin from heart muscle, according to our own measurements, has essentially the same molecular weight and shape as the skeletal protein, and the reactions with actin and ATP show no striking differences[28] Stern and his colleagues[29] have a different view but the discrepancy has not yet been satisfactorily resolved There are, however two points on which heart myosin differs from its skeletal counterpart First its ATPase activity is about one third that of myosin Second it appears to be much more resistant to trypsin and chymotrypsin It requires a level of the proteolytic enzymes about ten times higher to produce the same viscosity change In the ultracentrifugal sedimentation diagram, two major components appear but thus far attempts to separate and isolate them have not been successful.

Relaxation of models Glycerinated muscle fibers are as I mentioned earlier halfway between the isolated *in vitro* protein systems and the living muscle Weber and his colleagues have furnished, I think, very clear evidence for the close relation between ATP splitting and tension development in these fibers. They have shown that various factors that affect the rate of ATP splitting also affect the development of tension. This has been demonstrated for a wide variety of factors such as temperature changes, ATP concentration, and sulfhydryl reagents it has also been shown[illegible] that there is a close parallelism between the rate of splitting and tension development for a wide variety of nucleotide triphosphates

An important development was recognition of the fact that there are certain components in muscle that can effect relaxation of the glycerinated muscle fiber[illegible] The observations of Marsh[illegible] and Bendall[illegible] were the first ones to suggest that muscle itself contains a relaxing system. Subsequent work by Goodall and Szent-Gyorgyi,[illegible] and Lorand[44] appeared to pinpoint this relaxing factor in the creatine phosphokinase system and Moos and Lorand[illegible] later succeeded in showing that the phosphoenol pyruvate-pyruvic kinase combination can also act as a relaxing factor The possible role of myokinase has been suggested by Bendall[illegible] All of these systems are phosphor transferring enzymes. For the first two the phosphate donors are creatine phosphate and phosphopyruvate, respectively for the last the phosphate donor is ATP The phosphate acceptor is ADP in all three cases These systems resemble the original Marsh factor so named by Bendall,[illegible] in their requirement of Mg^{++} ions for relaxing activity and in being inhibited by Ca^{++} ions It was very tempting to speculate that these systems somehow act through the rephosphorylation of ADP bound to the muscle structure but all attempts to demonstrate a mechanism of this type failed

Another feature that has been demonstrated for most of these systems is the fact that concomitantly with the relaxing effect there occurs an inhibition of the ATPase of the fibers.[illegible] This appears to be consistent with the idea elaborated by Weber mentioned earlier that tension development and ATPase activity go hand in hand

The first suggestion that a simple phosphate transfer is not all that might be involved came from Kumagai and his colleagues,[illegible] who showed that they needed at least two protein fractions to achieve a relaxing effect One of these contained myokinase the other appeared to be similar in its sedimentation characteristics to the particulate ATPase of Kielley and Meyerhof[illegible] This observation has recently been amplified by Portzehl[illegible] and Lorand *et al*[illegible] However Briggs and Portzehl[illegible] have what to me appears to be good evidence that the need for a rephosphorylating system arises from the fact that most investigators of this problem have used rather thick fiber bundles. In this case the ATP concentration in the core of the fiber would be much lower than that in the peripheral parts and in the bath It has been shown by Weber and Hasselbach[illegible] that the relaxing effect depends on a certain critical ATP concentration below which there is no relaxation. Thus, according to Briggs and Portzehl[illegible] the rephosphorylating systems serve to raise the ATP con-

centration in the central portion of the fiber to the required critical level. It appears that in single fibers such systems as creatine phosphokinase or myokinase have no relaxing effect; in fact, they increase the tension if the ATP concentration is very low.

Briggs has now returned from Heidelberg to Harvard University and, recently, Kaldor and I have collaborated with him in our efforts to bring more light to this problem of the relaxing factor.[35] Using single fibers, we came to the conclusion that the relaxing system consists of at least two components, neither of which is any of the transphosphorylating enzymes mentioned earlier. By centrifuging fresh muscle extract prepared with a sucrose-buffer solution at 35 000 g, a fraction is obtained that by itself has no or only little relaxing ability. If, however, the supernatant is added, the fiber relaxes. If the supernatant is dialyzed, the dialyzate will also have the ability to supplement the particulate fraction (TABLE 4). The complete muscle extract contains large amounts of the granular portion, for it is possible to take about one third of the amount present in the originally active extract and activate it with an amount of the dialyzate corresponding to the full volume of the original extract. The dialyzable component appears to be a fairly labile substance; another clue to its nature may be found in the fact that it is readily absorbed on charcoal.

The fact that we were able to separate the relaxing system in these two factors has also opened up interesting possibilities for gaining further insight into the relationship of ATPase and relaxation. Although larger amounts of granules inhibit myofibrillar ATPase, with smaller amounts the cofactor requirement can be demonstrated also with the ATPase system (TABLE 5).

From time to time pyrophosphate (PP) also has been implicated in the relaxing mechanism, particularly by Bozler,[36] Weber[] and Bendall.[37] In our somewhat cleaned-up system we were able to show that PP in concentrations

TABLE 4

EFFECT OF THE TWO COMPONENTS OF THE RELAXING SYSTEM ON ATP INDUCED ISOMETRIC TENSION OF GLYCERINATED SINGLE FIBERS

Addition	Percentage decrease in tension
Crude extract, 1 ml	50
Granules, equivalent to 1 ml crude extract	5
Supernatant, 35 000 g, 1 ml	6
Granules and supernatant	41
Dialyzate of supernatant	7
Granules and dialyzate	47

Single fibers (60 μ thick) prepared from rabbit psoas muscle that had been extensively salted with 50 per cent glycerol and stored at −15° C. The bath contained 2 mM Mg^{++}, 5 mM ATP, 0.02 M Tris buffer, pH 7.5; the ionic strength was adjusted with KCl to 0.16, total volume 4 ml. The "crude extract" was prepared by blending rabbit muscle in 3 volumes of 11 per cent sucrose, 0.02 M histidine, 0.06 M KCl, 0.0025 M oxalate (pH 7.5) and centrifuged at 3600 g. The supernatant is referred to in the table as "crude extract." Further centrifugation followed at 35,000 g, and the sediment is referred to below as "granules." The granules were taken up in 11 per cent sucrose in one fifth of the original volume of the extract.

TABLE 5

EFFECT OF THE "RELAXING" SYSTEM ON MYOFIBRILLAR ATPASE

Additions	Percentage inhibition of phosphate liberation
Granules, 0 1 ml	77
Granules, 4× dil 0 1 ml	0
Granules, 4× dil 0 1 ml + 2 mM PP	81
2 mM PP	0
Granules, 4× dil 0 1 ml + 0 1 ml dialyzed 35,000 g supernatant	60
0 1 ml supernatant	0

The myofibrillar suspension was prepared by homogenizing in the Waring Blendor rabbit skeletal muscle with 5 volumes of 0 065 M succinate buffer pH 7 5 followed by centrifugation at 1600 g The sediment clearly showed two layers the bottom layer contained unbroken cells and connective tissue debris, the upper layer containing the myofibrils, was resuspended in the buffer and the centrifugation-resuspension procedure repeated 3 more times The final fibril suspension contained 1 6 mg of N/ml The "granules" and supernatant were prepared as described in the legend of TABLE 4 The "granule" suspension contained 1 8 mg N/ml

The ATPase assay was carried out in the medium used for the preparation of the granules in total volume of 1 ml containing 5 mM of ATP and 5 mM of MgSO The basic system contained 0 1 ml of myofibrillar myosin Other additions are as indicated in this table The maximal phosphate splitting amounted to 1 1 μM in 5 min incubation at 23° C Inorganic phosphate was determined after deproteinization with trichloracetic acid according to the Fiske Subbarow method

of about 2 mM does not by itself produce relaxation. If however PP is added to the granules, it can replace the dialyzable factor Clearly however it is not the active substance of the dialyzate in view of the lability of the actual dialyzable component and its absorption on charcoal Moreover inorganic PPase inhibits PP relaxation in the presence of granules, but leaves the dialyzable component from muscle fully active PP has a similar effect on the ATPase of muscle fibrils in that concentrations of PP that by themselves have no effect on the ATPase activity become inhibitory in the presence of such amounts of the particulate fraction that by themselves do not inhibit the ATPase (TABLE 5)

With this relaxing system, as with the muscle proteins I discussed earlier the question of its physiological significance always looms large in our minds As we gain more insight into the working of this relaxing system we shall want to know does it work in the intact muscle how is it incorporated into the general scheme of excitation and relaxation of the development and the decay of the active state? We should like to know how it is localized in the muscle that is the same question we ask in connection with those muscle proteins about which we have much more detailed knowledge at this time However while we cannot answer these questions with respect to the relaxing factor I think we have much more information about the structural proteins discussed earlier

Muscular structure and proteins As regards the problem of the localization of the structural proteins in the muscle fiber the model developed by Hanson and Huxley[87] seems to be supported by the evidence although some

investigators, including Hodge [*] think that other models are more plausible. Podolsky elsewhere in this monograph has shown that either the "classical" model involving shortening of protein filaments, or the "interdigitating" one based on the relative motion of protein chains whose configuration does not change, could account for the rate of energy liberation observed by Hill.

The proponents of both types of models have assumed that myosin and actin as we know the proteins in solution, also exist in the muscle fiber. Holtzer and Marshall[*] recently reported some experiments with fluorescent antibodies that at first glance might render this supposition untenable.

These investigators prepared fluorescent antibodies against the so-called meromyosins and found that the antibody against the heavy component reacts only with the middle of the A band, the so-called H zone, whereas the other appears to be bound to the rest of the A-band. I do not believe that it can be said at this stage that this is evidence for the existence in intact muscle of these units smaller than myosin. If the components reacting with the antibodies were indeed, closely related to the fragments against which the antibodies were obtained and if, indeed, the smaller units existed in muscle in widely separated regions, it would be necessary to discard our whole concept of the myosin molecule. This is all rather new material and I do not think that we have sufficient evidence for a truly critical discussion, but I suggest that it is quite conceivable that the structural relationships existing in muscle may interfere with the reaction of the antibodies and proteins in such a way that the resulting pattern is the one described by Marshall and Holtzer.[*]

It may be more than coincidence that the anti-H-meromyosin reacts only in that part of the sarcomere where, according to the Huxley Hanson[*] picture there is no actin. Remembering that H-meromyosin reacts *in vitro* with actin, it may be possible that in the rest of the A-band this reaction prevents the combination with the antibody.

I would urge great caution in the use of terminology. Meromyosin has become a generic term for various fragments produced from myosin by proteolytic enzymes. Strictly speaking, these fragments cannot be identical with anything in muscle because of the chemical changes brought about by the proteolytic enzymes. Thus, if units smaller than myosin exist at all in intact muscle they cannot be identified with any kind of meromyosin; consequently the binding to muscle of antibodies against the proteolytic meromyosins cannot directly be used as a means for locating structural components. This is not mere quibbling. I feel strongly that to disregard these points is quite possibly to introduce artificial complications into the already complicated picture.

If we do accept, at least for the purpose of argument, the model put forward by Huxley and Hanson,[*] we must regard muscle as a contracting system both in terms of our knowledge of the soluble components *in vitro* and in terms of the possibilities afforded by the structure of the muscle fiber. Theories of muscle contraction in the past have usually considered the contraction as based on some changes in the actomyosin molecule or, if some authors have not been quite as explicit as that, they certainly have considered changes in the shape and configuration of some molecules as responsible for contraction.

For some time, however one difficulty has been pointed out that is, the fact that in X ray studies no significant changes could be detected among relaxed contracted and stretched muscles. If a gross coiling or uncoiling occurs in the contraction cycle it should be visible in the diffraction diagrams.[69] However the model proposed by Huxley and Hanson produces an entirely different situation. Here we have a longitudinal displacement of molecules with respect to each other without permanent changes in the molecular configurations of the molecules themselves In a recent review A. Huxley[60] has explored the implications of the Huxley Hanson model and has attempted to show that such a model would lead to consequences consistent with the known properties of muscle as a whole elsewhere in this monograph Podolsky discusses some more general features of thermodynamic theories of muscle contraction. I now propose to discuss the implications of the Huxley Hanson model in terms of known chemical and enzymatic properties of the muscle proteins

It appears to me that a contraction phase based on the combination of myosin and actin involving sulfhydryl groups of myosin would be consistent with the known tendency of myosin and actin to combine. If as I earlier suggested one could take the reaction of actin and myosin in solution with each other and with ATP as an indication of what happens in muscle, one would see that, if ATP was somehow allowed to combine with the relevant site, it would cause dissociation and, hence, in terms of this model relaxation As long as ATP could be present around the sites involved in the combination with actin, the actin and myosin would remain dissociated and no contraction would occur There is good correlation between the effect of sulfhydryl reagents on the tension exerted by glycerinated fibers and the requirements of this picture in which the blocking of such sites would be expected to lead to dissociation and relaxation The relaxing system, by inhibiting ATPase activity tends to increase the number of ATP molecules near the sites involved both in splitting of ATP and in the combination with actin The persistence of ATP in the relaxed state would also be favored by the fact that in the presence of magnesium, myosin dissociated from actin has very low ATPase activity

Energy source of muscle contraction I now turn to some problems of the energetics of muscle. The first question that comes to mind is this Is ATP involved in the elementary event of muscle contraction? Answers to this question have generated considerable interest, some heat, and possibly some light, thanks to the work of Mommaerts, Fleckenstein, Krebs, and others [61, 62, 73] Their work suggests, *prima facie* that during a single twitch or even during a short tetanus, no detectable changes occur in ATP ADP inorganic phosphate, or creatine phosphate. In my own mind there is no doubt that Mommaerts [64, 65] careful experiments have shown that, as far as ATP and ADP are concerned one can unhesitatingly say that under conditions where rephosphorylation both aerobic and anaerobic, is not inhibited no changes occur Owing to technical difficulties I am not certain that one can say that the data really exclude changes in the creatine phosphate level since the total expected change would be about one quarter to one half μM/gram of muscle

and the total creatine content is 10 t 20 μM The data of Fleckenstein and his colleagues,[29] due to their use of a different technique, make the lack of change in the creatine phosphate level more convincing. However in neither of these experiments was the glycolytic cycle inhibited, and I think it would be extremely hazardous to say that phosphate transfer could not have occurred. Another difficulty with all experiments of this type is the method of fixation. Again, I think Mommaerts has probably gone as far as possible in making sure that muscles do not ontract at the moment they are fixed by freezing However it is one thing to say that there is no visible contraction and quite another to assume that no hemical a tivation leading to changes in the phosphat distribution has oc urred Spectroscopic measurements of the type carried out by Chanc and his co-workers[30, 31] may eventually throw more light on this question although at the moment their experiments are not conclusive

There is no question that in the longer series of twitches ATP does disappear We could cite the classic work of Lundsgaard and, more recently the work of the Fleckenstein *et al* [29] Recently both Caspó and myself and also Carlson *et al* [32] have shown that the first substance to show changes is creatine phosphate, and there is a fairly good linearity between the duration of stimula tion and the changes in the creatine phosphate level Therefore by using the mathematical argument of continuity it would seem that one could define an amount of creatine phosphat corresponding to a single twitch and that one ould argue also that the same amount must disappear in the first twitch of the series, since no break in the curve is d tectable

It seems to me that whether ATP disappearance can be shown in a single tw tch is hardly one of the most fundamental questions Assume that there is an unknown, or unsuspected substance X or XP that plays an important part in the ontraction It may act either directly or indirectly If directly ATP may be able to rephosphorylate this substance very rapidly so that even in the first twit h of a series a change would be detectable Alternatively it may not be able to rephosphorylate apidly in which case the first twitch or t o perhaps, will not be accompanied by changes in any known substance However this situation could obtain if ATP reacted immediately and if in turn t was rephosphorylated very rapidly Therefore I think that the rucial question is one of mechanism and not of stoichiometry and that balance studies an serv only as landmarks in exploring the unknown territory of the chemical mechanism of ingl twitch

For the purpose of th follow ng discussion I shall assume and I think most people will agree, that the ultimat source of th energy of muscle contraction consists of some high-energy phosphates, the bulk of which are present as ATP and creatine phosphate In the most general terms this problem of energy utilization is one of thermodynamics, in the sense that the first law of thermodynamics will not permit us t produce mor work than ill correspond to the total chemical energy present the second law ill impose further limita tions in terms of efficiency in that only the free energy of a chemical reaction is ava lable for mechanical work Since eventually the liberation of energy

from the high-energy phosphate compounds involves the hydrolysis of one or more phosphate bonds, the obvious thing to do is to look for a system that can split phosphate bonds. Most people working in the field of muscle research have concluded not unnaturally that the ATPase activity of myosin and actomyosin cannot be considered as accidental but that they must have an important role in the liberation of energy for contraction. Certain difficulties have been raised in this connection by Mommaerts,[96] who has pointed out that the ATPase activity of myosin is too low to account for the phosphate liberation in living muscle. However the recent work of Morales and his colleagues[97] has shown that substances that apparently have no known role in the metabolism of muscle, such as aminoethylisothiouronium can considerably increase the ATPase activity of muscle. Therefore, by assuming the existence of similar unknown activators in muscle or by assuming that the structural relations have significant effects on the ATPase activity the gap could be considerably narrowed. In connection with the latter point it should be noted that the activity of purified cytochrome c is much lower in a reconstructed system than in the intact system.[98]

The "how" of the conversion of the phosphate bond energy is of the greatest theoretical interest in the problem of what might be called chemicomechanical coupling. The theories could be divided into two groups: classical ones and perhaps, those that might be called quantum-mechanical theories. In the latter group Albert Szent-Gyorgyi is one of the pioneers.[2, 99] Of course, it should be emphasized that even the "classical" chemists of today are cognizant of the use of quantum mechanics: the most classical-looking organic chemical reaction can be described in terms of delocalized orbitals within various organic molecules. The effect of certain substituents on the reactivity of aromatic compounds has been most elegantly discussed, for example by Longuet Higgins.[100] For the purpose of this discussion however we can make use of concepts such as group transfer, charge effects, change in shape due to entropic factors, and others. Szent-Gyorgyi[2] advocates the consideration of the possibility of energy transfer throughout the biological fabric similar to that occurring in an electric conductor or semiconductor with an electron gas. If the structural views suggested by the experiments of Holtzer and Marshall[68] were to prevail, some such mechanism would be almost inevitable since the ATP splitting would occur in spatial separation from the rest of the contractile mechanism. Even in this case one would eventually find it necessary to make use of the classical concepts in describing the actual mechanism of contraction. Just as, in describing the working of an electric motor for example, in which the transfer of energy in the wire proceeds according to the quantum mechanical concepts of the electron gas, use must be made of classic mechanical concepts and devices, such as momentum, gears, and axles. It is possible however that in muscle we may eventually arrive at a description that can dispense with all localized concepts such as shortening of bonds, folding of molecules, and shifting of chains and, as Szent-Gyorgyi[2] suggests, there may be a wholesale change in the water structure so that the whole structure could collapse if the energy somehow either leaked out or were put into it.

I think that here we have reached the limits set by the principle of complementarity and that only time will indicate our future course. In the coming years the values of some of our observables, using the language of quantum mechanics, will have changed from unsharp to "sharp and whereas today we must speak in terms of a probability distribution of theories, we may then arrive at a ertainty orresponding to an *eigenstate*

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MYOCARDIAL EFFICIENCY

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Faithful adherence to the topic of heart efficiency would lead to a discussion of dynamic events, such as resistance, flow and pressure. The following paragraphs, however, are restricted t review of two factors that influence the calculation of myocardial efficiency: anaerobic metabolism (glycolysis) and oxygen consumption of the nonbeating heart. The final portions of the paper are devoted t discussion of myocardial energy utilization.

The efficiency of the heart can be determined by the ratio of the thermal equivalent of the work performed t the heat liberated. Therefore, it can be indirectly calculated by comparing the work performed with the myocardial oxygen usage. Th former is synonymous w th energy utilization, the latter represents total energy production. Determinations of myocardial efficiency from th oxygen consumption of the heart cannot be made unless one is sure that anaerobic glycolysis in heart muscle is absent. An additional difficulty in the calculation of myocardial efficiency arises from the fact that the calculations are valid only as long as the energy produced, aside from being degraded into heat, is utilized exclusively for "useful work." This represents only the mechanical work expended by the heart in overcoming resistance in the systemic and pulmonary circulation and in imparting momentum to the blood stream. Therefore a knowledge of the oxygen consumption of the non-beating heart is essential in th determination of myocardial efficiency. I the presence of constant external work, the efficiency of the beating heart depends t a large extent on the oxygen consumption of the arrested organ.

Catheterization of the coronary sinus has made it possibl to study the metabolism of the heart in closed-chest animals and in man. These studies have indicated that fatty acids furnish the greatest contribution to the total energy production of th heart. In the fasting state, the relationship between the contribution of carbohydrates nd non-carbohydrates to total energy production is bout 1 to 3. The heart can use all substrates circulating in blood, and in general the amount of y individual substrat utilized depends on its rterial concentration. Storage of foodstuff by the heart occurs; f example carbohydrates are stored as glycogen and fatty acids as neutral fat. There is no evidence of glycolysis in the normally beating heart in situ. Under certain abnormal conditions, however glycolysis of the heart muscle may play a role in energy production. According to expectation, this occurs when the oxygen supply to th heart muscle diminishes, as for example in myocardial infarction and following arrest of the coronary circulation. Under these conditions, lactate and pyruvate concentrations in coronary vein blood exceed those in arterial blood. Anaerobic glycolysis is reversible, provi

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TABLE 1

Preparation	Q_{O_2} (Left ventricle)
Tissue slices (rat)[a]	3 5
Arrested dog heart[a]	10 0
Beating dog heart	54 0
Beating human heart	25 4

the period of ischemia and anoxemia is not too extensive (less than 2 to 3 hours)

The second factor that plays an important role in determining myocardial efficiency is the oxygen consumption of the arrested heart It has been demonstrated that the oxygen usage of the heart arrested with potassium chloride varies between 20 to 35 per cent of that of the naturally perfused heart *in situ*[4] This finding is in agreement with results obtained by Gregg and Sabiston[6]

A comparison may be made between the O consumption of tissue slices and that of the whole arrested heart *in situ* This necessitates conversion of myocardial O_2 usage into units of dry weight, which can be accomplished by a factor 5 22 representing the relationship between dry and wet weight of tissue slices. The results of these calculations are presented in TABLE 1 Studies from this laboratory have shown that there is no significant difference between *the oxygen consumption of the heart beating with empty ventricles, the* fibrillating heart, and the heart arrested with potassium chloride[4] In contrast Lorber[7] has found the oxygen consumption of the fibrillating heart to be greater than that of the arrested or of the normally beating heart It is possible that the high myocardial oxygen usage reported by Lorber results from the fact that in his preparation the chambers of the heart are filled with blood therefore, the tension and length of the muscle fibers are greater than in the experiments reported from this laboratory where the cardiac chambers were empty[4] The relatively high oxygen consumption of the arrested perfused heart necessitates a re-evaluation of previously published data on myocardial efficiency Such calculations can now be made from the difference in myocardial O_2 usage between the naturally beating and the arrested dog heart (mean O_2 usage of the beating heart is 25 11 ml /min mean O_2 of the arrested heart is 4.83 ml./min.) Consequently the O_2 consumption of the latter is 19 4 per cent of that of the beating heart Accordingly 19 4 per cent of the total O_2 consumption of the left ventricle is used for basal metabolic processes exclusive of work. The efficiency of muscular activity of the heart can therefore be obtained as follows

$$\frac{\text{Work of heart}}{O_2 \text{ consumption /min.} \times 2.059 \times 0.806}$$

where 2 059 is the energy equivalent (kg ml) of 1 ml of O_2 at an *RQ* of 0 82 and 0 806 is the fraction of O_2 used in the contractile work of the heart only According to these calculations, myocardial efficiency is approximately 37 per cent in the dog and 39 per cent in man

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